Studies on barley malt kernel heterogeneity

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Abstract

Standard analysis of malt flour can mask the heterogeneity of hydrolytic enzyme activity. Kernel heterogeneity can lead to brewhouse problems and a product with unpredictable nitrogen and fermentable sugar content. The variability between individual grains of important malting characteristics was measured in malt samples produced under aerobic and anaerobic conditions.

Various parameters (including germinative energy, germinative capacity, moisture content, β-glucanase activity, friability and homogeneity) were measured to ensure that the 5 d aerobic *Optic* malt, produced in Heriot-Watt university micromaltings, was viable and of commercial quality.

The 5 d aerobic malt kernels produced at Simpsons Maltings in Berwick-Upon-Tweed were heavier than the micromalt. Commercially produced malt kernels had higher levels of fermentable sugars and soluble nitrogen than the micromalt despite lower α-amylase, β-amylase and 'total' limit dextrinase activity.

Differences between the 5 d aerobic micromalt and the 5 d aerobic commercially produced malt are indicative of why micromalting cannot always be used as a model system for what is happening industrially and must be modelled on commercial practice.

Subjecting 5 d aerobic micromalt to 24 h anaerobic incubation resulted in increased levels of fermentable sugars per l wort. 24 h anoxia also resulted in increased α-amylase and limit dextrinase activities. There are potential industrial applications for this anaerobic wort.

Limit dextrinase inhibitor protein present in crude extract prepared from mature barley, eluted from a gel filtration column at a higher molecular weight than expected. The limit dextrinase inhibitor protein either aggregates or binds to other proteins in a high molecular weight complex.

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Chapter 1. Introduction

1.1. Brewing and distilling

The dictionary defines brewing as “the making of beer or related beverages by infusion, boiling and fermentation” and distilling as “the production of whisky or spirits by heating the liquid until it evaporates and cooling it again until it becomes liquid”. Brewing and distilling form the basis of a massive global industry, $1.5x10^7$ tonnes of malted barley are produced every year, 94 % of which is used in brewing and 5 % in distilling (Anderson, 2000). The conversion of starch to fermentable sugars is key to the production of cereal based alcoholic beverages. A great deal of economic advantages can be gained by having an increased control of starch processing and any factors which limit the fermentability of the end product (Anderson, 2000). Understanding malt enzyme expression, activation and regulation is essential in developing new varieties of crops containing improved characteristics, yields or processing properties.

1.1.1. Steeping

In the malt house the grain imbibes water, via the micropyle and then the embryo during controlled cycles of water, spraying or immersion followed by aeration (to ensure that the grain is not asphyxiated and to reduce water sensitivity which often inhibits germination) until the moisture content of the grain reaches 38 to 40 % (w/w) (Brookes *et al*., 1976). Regimes of submersion and air rest periods reflect individual barley sample requirements, water availability and the production cycle; understeeping can often result in limitation of endosperm modification and thus reduce malt quality (Palmer, 2006). Steeping washes out a wide range of materials including phenols, amino acids, sugars, minerals and microorganisms (Palmer, 2006; Hough, 1991).

1.1.2. Germination

Once grains have chitted (the first visible sign of germination is the appearance of the white root or ‘chit’) they are laid on a shallow malt bed for a period of around 5 days and allowed to germinate (producing shoots and roots), chilled moist air is passed through the bed to ensure that the grain is cool and moist (Eaton, 2006). The grain bed is rotated regularly with a rotating screw to prevent the grain roots and shoots from matting together. Optimal moisture levels of ~45 to 46 % (w/w) are required for optimal initiation of endosperm modification (Palmer, 2006). During the first 2 days of

germination the plant releases hormones such as gibberellins which trigger the production of enzymes in the aleurone layer; enzymes are released from the aleurone layer and join enzymes already present in the grain. Enzyme activities reduce the viscosity of solubilised β-glucan material and hydrolyse starch into fermentable sugars (Palmer, 2006; Hough, 1991).

1.1.3. Kilning

If allowed to continue to germinate a new plant would be formed but, the maltster halts germination by heating or kilning the grain with hot dry air, this drying of the grain not only acts to preserve it but also contributes to colour and flavour development in the beverage (Eaton, 2006; Palmer, 1989). Kilned malt typically has a moisture content of 4 to 5 % (w/w) and is thus stable for storage (Briggs, 1998; Palmer, 1989). 'Green malt' (unkilned malt) is used directly in some distilleries as it has 30 to 50 % greater enzyme activity, such malts may however contain higher levels of S-methylmethionine and produce high levels of dimethylsulphide which can impart extra sulphurous notes to the whisky (Prentice *et al*., 1998). The other disadvantage to using 'green malt' is that it cannot be stored due to continuous growth which can lead to malt stewing and as a result quality deterioration. Despite reduced kilning costs 'green malt' is also very difficult to transport.

Kilning occurs in stages, firstly an enzymatic stage - in which the moisture content is reduced to 10 % (w/w) and the air-temperature does not exceed 50 ^{o}C and the enzymes retain activity. A physiochemical stage follows whereby the moisture content is decreased further to 4 to 5 % (w/w); the air temperature varies from 50 to 75 ^{o}C decreasing enzyme activity substantially (Bathgate, 1989).

1.1.4. Mashing

Kilned malt must then be milled into smaller particles called grist. Milling makes the grain easier to wet and aids faster extraction. Roll mills produce coarse grist for use with mash or lauter tuns but, mash filters can use finer grist produced by hammer tuns (Eaton, 2006). At this stage raw pre-cooked cereal adjuncts (i.e. barley, oats, wheat, rice, rye and sorghum) can be added to the malt and liquor at temperatures of 62 to 65 ^{o}C. Adjuncts are usually added for one or more of the following reasons: to produce a more stable beer due to lower protein levels, to add flavour, to produce better beer

foam due to lower lipid levels, to ease brewhouse processing or to produce a beer at lower cost. In Britain infusion mashing or a classic thick mash system is used, the mixed grist and liquor are heated to ~ 62 to 65 ^{o}C for 90 minutes. This process allows for balanced gelatinisation of starch and α and β-amylase activity. Gravity (mash density) and pH of the mash are important in determining enzyme activity (Palmer, 1990). Higher gravity mashes have been reported to increase enzyme survival (Sissons *et al*., 1995).

In beer production wort is boiled to eliminate bacteria, yeast and mould which might compete with the yeast for nutrients and produce 'off' flavours. Wort boiling also removes any bitterness caused by hops (introduced early on in the boil), allows excess proteins and tannins to form solid particles or trub which is later removed (this is important for stability and foam), allows colour and flavour to develop, boiling also removes undesirable volatiles such as dimethyl sulphide by evaporation and concentrates sugars by removing water (Eaton, 2006).

In grain distilleries wort is produced by mashing 10 % (w/w) malt slurry mixed with 90 % cooked cereal at 65^{o}C for 90 minutes. Wort production in malt distilleries usually involves mashing with 3 sequential batches of water (at temperatures of 65, 75 and 85 ^{o}C) to maximise fermentability (Bathgate, 1989). In some distilleries a fourth water is used at a temperature of 90 ^{o}C (Wilkin, 1989). The wort used in the third and fourth waters (if carried out) is usually low in fermentable sugars and is used as the liquor source of the first water of the next batch (Bathgate, 1989). A key feature of wort used in malt distilleries is that it is unboiled and non-sterile, this ensures that enzymes which survived the mashing process are present and active during fermentation, this secondary conversion of dextrins into fermentable sugars increases the efficiency of starch degradation and increases ethanol yield (Hardy *et al*., 1989). In spite of secondary conversion up to 5 % of starch may be hydrolysed into non-fermentable branched dextrins (Palmer, 1989).

1.1.5. Fermentation

The handling of the yeast is key to efficient brewery fermentation and good beer quality. Yeast is pitched into the wort (following cooling to required fermenting temperature: 8 to 13 ^{o}C for lager, 14 to 17 ^{o}C for ale and in excess of 20 ^{o}C for

distillation) at a typical rate of 5 to 20 million cells per ml wort. Fermentation is allowed to take place whereby yeast utilise the sugars and nitrogen to produce CO_2, ethanol, new yeast cells and flavour compounds (Eaton, 2006).

1.2. Starch

Starch makes up 58 to 64 % of a barley grain's dry weight, it exists as large, insoluble granules which vary in size and shape with plant species and development (Manners and Stark, 1974). Starch is made up of two glucosidic polymers – amylose and amylopectin granules. Amylose is a linear molecule with a molecular weight of 10^5 to 10^6 Da. In barley amylose makes up ~30 % starch total, it consists of linear α-1, 4 linked glucan chains with less than 1% α-1,6 linked glucan branching. Amylopectin is the major starch component with a molecular weight of 10^7 to 10^8 Da it constitutes 70 % of starch granule mass. Amylopectin consists of α-1, 4 glucan chains interspersed with ~5 % α-1, 6 glucosidic linkages (Manners, 1974). Distribution of glucan chain lengths and branch point clustering allows the chains to form double helices which can pack together in organised arrays. These arrays form the basis of the semi-crystalline nature of much of the matrix of a starch granule (Ball *et al*., 1996). Starch crystalline structure is highly conserved in plants at a molecular and microscopic level where crystalline and amorphous material (growth rings) are present in all higher plant starches (Tetlow *et al*., 2004).

In the first 2 weeks of endosperm development of barley (which reaches maturity 9 weeks post anthesis), one starch granule is typically initiated per amyloplast; granules are lenticular in shape (Duffus and Cochrane, 1993; Buttrose, 1960). At maturity granules can be up to 35 μm in diameter; these large A-type granules cease to be produced after 2 weeks post anthesis (May and Buttrose, 1959). At this time smaller rounder B-type granules are produced in the stroma of A-type amyloplasts, they are then released into the cytoplasm in a double membrane budded off from the parent amyloplast (Buttrose, 1960). A-type granules range in size from 10 to 30 μm (they generally tend to be 15 to 20 μm in diameter) and despite making up a high proportion of total starch weight (85 to 90 %) they are only a small proportion of granule numbers (10 to 20 %) (MacGregor and Fincher, 1993). In contrast B-type granules are usually smaller than 6 μm in diameter and despite accounting for 80 to 90 % of granule numbers only make up 10 to 15 % of the total starch weight (MacGregor and Fincher,

1993). It has been suggested that large granules have a higher amylose content (M^{c}Donald *et al*., 1991; Stark and Yin, 1986).

A cluster model has been proposed for amylopectin, the general consensus is that amylopectin is made up of three different types of glucosyl chains: A, B or C (Figure 1.1). A-chains are the outer side chains, they are unbranched and linked by their potential reducing group to the main chain with a α-1, 6 branch. B-chains are branched as they are capable of binding to other glucosyl units through their C_6 side chain containing the reducing end; there is only one of these per amylopectin molecule (Nikuni, 1969; French, 1972; French, 1984; Hizukuri, 1986; Robin *et al*., 1974). Hizukuri (1986) proposed that B chains which are present in a single cluster are designated B1 chains, longer chains interconnecting the clusters are referred to as B2 or B3 chains, depending on the number of clusters they span (figure 1.1). Crystalline regions of starch are thought to be formed by A and B1 chains intertwined into double helices, α-1, 6 branches are arranged ensuring that at 9 nm intervals along the molecule axis there are chain clusters containing between 12 and 16 glucosyl units (Smith *et al*., 1997). Crystalline lamellae are the result of adjacent cluster branches forming double helices which alternate with branch point containing amorphous lamellae every 9 nm along the molecule axis.

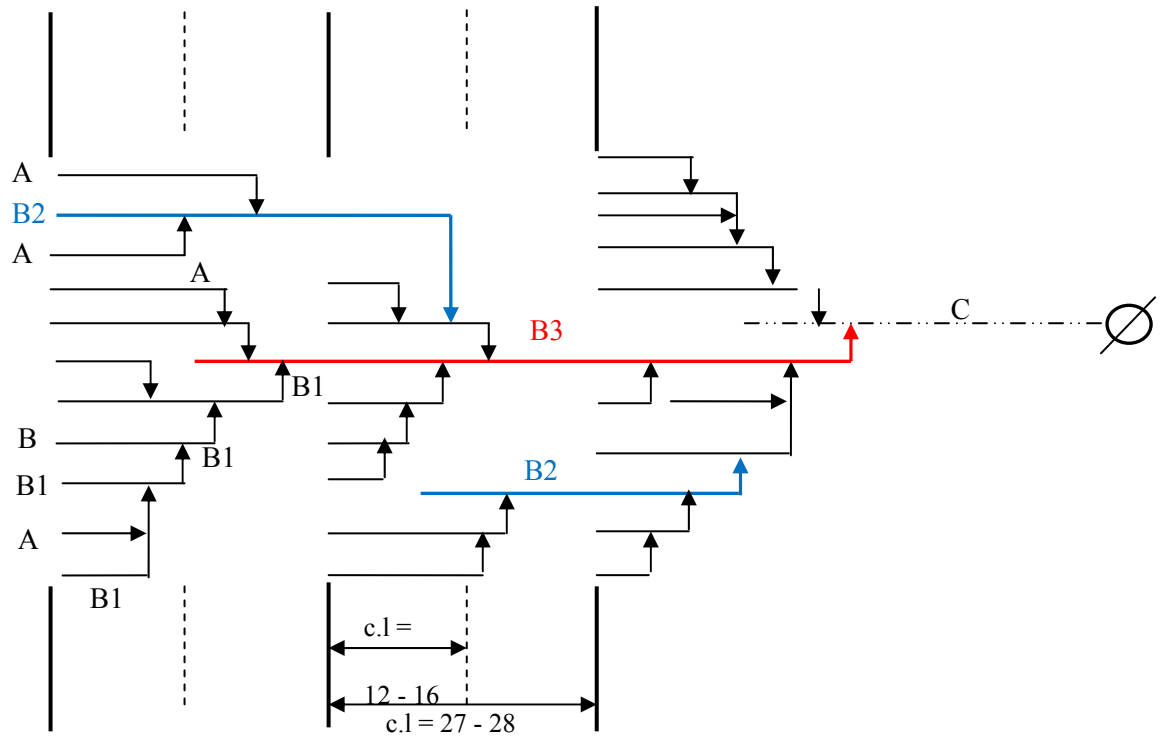

Figure 1.1: A cluster model for amylopectin, adapted from Hizukuri, 1986

∅ Shows the position of the reducing chain end

—— Shows the position of α-1, 4 glucan chains

→ Shows the positioning of α-1, 6 glucan chains

C.l Gives an indication of the chain length (glucosyl units).

1.2.1. Starch biosynthesis

During photosynthesis sucrose is produced in the leaves and transported to starch storing organs via the phloem where it can be converted to starch. According to the Münch – Horwitz hypothesis described by Goeschl and Magnuson (1986) solution flow through the phloem occurs down a pressure gradient from the source (photosynthetic tissue) to the sink (i.e. fruit, seeds, root/shoot meristem). Hydrostatic pressure is generated at the source in the collection phloem through solute uptake into the sieve element/companion cell complex. In addition to sucrose the solute contains other sugars (in lower concentrations), amino nitrogen compounds and potassium (Smith *et*

al., 1980; Lalonde *et al*., 2003). Solute appears to be actively loaded from the apoplast to the sieve element/companion cell complex against a concentration gradient by H^+ ATPase (Patrick *et al*., 2001). Solute uptake creates a water potential gradient between the apoplasm and the sieve element/companion cell complex, down which water flows into the sieve element/companion cell complex generating the hydrostatic pressure gradient between the source and sink phloem which drives solution flow. A lower pressure is maintained in the release phloem during solute unloading (Gould *et al*., 2005). Phloem unloading has been extensively studied (reviews by: Oparka, 1990; Patrick, 1990; Patrick, 1997; Fisher *et al*., 1996; Schulz, 1998) it is however still poorly understood. During phloem unloading, assimilates carried by mass flow exit the sieve element/companion cell complex and are transported through a diverse range of non-phloem tissues (Oparka, 1990; Patrick, 1990). Assimilates may leave the sieve element/companion cell complex via two potential pathways: through plasmodesmata into vascular parenchyma elements (symplastic unloading) or directly across the plasmalemma to the apoplast (apoplast unloading), it is also conceivable that both pathways operate simultaneously (Oparka, 1990). The vascular bundles of the barley leaf display two distinctive types of sieve element, thick walled sieve elements (which lack companion cells) (Evert *et al*., 1996) and thin sieve elements which are typically connected to companion cells by pore-plasmodesma units (Van Bel *et al*., 1997). Based on an ultrastructural study of plasmodesmatal frequencies in the barley leaf blade carried out in 1996, Evert *et al*. concluded that apoplastic phloem unloading was utilised by the barley leaf. This theory was contradicted in 2001 by a study conducted by Haupt, *et al.* which provided evidence of symplastic sieve element unloading which implicated both thick and thin sieve elements.

The first committed stage of starch biosynthesis (Figure 1.2) involves AGPase (Adenosine 5' diphosphate glucose pyrophosphorylase, E.C.2.7.7.27), which is responsible for the production of ADPglucose, an ADPglucose transporter is then responsible for targeting it to the amyloplast (Smith, 2001). AGPase is heterotetrameric, composed of two large (AGP-L) and two small (AGP-S) subunits (Preiss *et al*., 1996). At least two distinct AGPase enzymes have been identified in the barley endosperm (Thorbjørnsen *et al*., 1996); they have been shown as plastidyl and cystolic isoforms, the developing endosperm cystolic isoform accounts for 65 to 95 % of the total activity. The mRNA of plastidyl and cystolic AGPase of barley are produced from a single gene by using 2 alternate first exons (Thorbjørnsen *et al*., 1996). The role of cytoplasmic AGPase is unknown, it may contribute to ADPglucose to starch synthesis. The plastidial AGPase isoform is probably capable of catalysing the entire

flux of carbon to starch (Thorbjørnsen *et al*., 1996). Once inside the amyloplast starch synthases (SS - E.C.2.4.1.21) catalyse the transfer of ADPglucose to the reducing end of the pre-existing α-1,4 linked glucan primer, thus elongating glucan chains and synthesising amylose and amylopectin.

Group 1 starch synthase genes (Granule bound GBSSI and GBSSII) are encoded by the waxy locus and function specifically to elongate amylose chains by the formation of α-1, 4 linkages (Nelson *et al*., 1962). GBSSI expression is confined to the matrix of the starch granule and has also been associated with amylopectin elongation, its precise mechanisms for synthesising amylose and elongating amylopectin are unknown (review by James *et al*., 2003). GBSSII is encoded by a separate gene and is responsible for amylose extension in leaves and non storage tissues (Nakamura *et al*., 1998; Vrintin *et al*., 2000). GBSSI activity is stimulated by malto-oligosaccharides, which diffuse into the granule matrix where they act as primers elongation by GBSSI (Wang *et al*., 2001; Denyer *et al*., 1996).

Group 2 starch synthase genes (SSI, SSII, SSIII and SSIV) are exclusively involved in the production of amylopectin. SSI is responsible for short glucan chains of DP <10 (Commuri and Keeling, 2001). SSII has two isoforms, SSIIa is present in cereal grain endosperms and synthesises glucan chains of intermediate length of DP 12 to 24 by elongating short chains of DP <10, the down regulation of SSIIa has a dramatic impact on starch amounts and composition despite only accounting for a small proportion of total SS activity (Imparl-Radosevich *et al*., 2003).

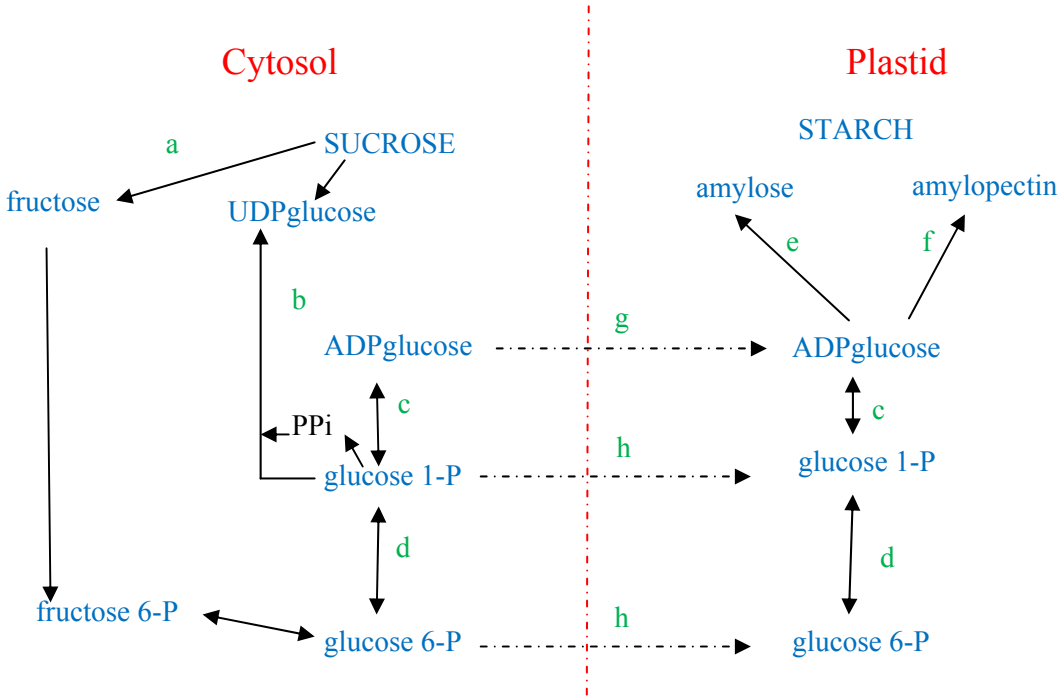

Figure 1.2: The conversion of sucrose to starch in storage organs – a to h represent enzymes involved, PPi indicates inorganic pyrophosphate.

a. sucrose synthase
b. UDP glucose pyrophosphorylase
c. ADp-glucose pyrophosphorylase
d. phosphoglucomutase
e. granule bound starch synthase I
f. starch synthase and starch branching enzyme
g. ADP glucose transporter
h. hexose phosphate transporter

Starch branching enzymes (SBE, E.C.2.4.1.18) are responsible for the generation of α-1, 6 linkages, by cleaving internal α-1, 4 bonds and transferring the released reducing ends to C6 hydroxyls forming the branched structure of amylopectin. SBE activity is a function of multiple isoforms, SBEI and SBEII are the two major classes which differ in substrate specificity and the length of glucan chain transferred (SBEII favours short chain glucans). The N and C termini of the proteins play an important role in the determination of substrate preference, catalytic capacity and chain length transferred (Takeda *et al*., 1993; Guan *et al*., 1993; review by Myers *et al.,* 2000).

The accumulation of water soluble polysaccharide (phytoglycogen) in low starch mutants lacking debranching activity implicates isoamylase-type debranching enzymes (E.C.3.21.1.68) and pullulanase-type debranching enzymes (limit dextrinase E.C.3.2.1.41) in starch biosynthesis. Typical roles of debranching enzymes are the hydrolysis of α-1,6 glucosidic linkages, debranching enzyme activity in the starch biosynthetic pathway have yet to be determined, models have however been proposed.

Isoamylase-type debranching enzymes cleave α-1,6 branch points in amylopectin and glycogen, they are incapable of hydrolysing α-1,6 bonds in pullulan (an α-1,6 linked maltotriose polymer). Pullulanase type debranching enzymes (also referred to as R-enzymes or limit dextrinase are capable of cleaving α-1,6 glucosidic linkages in pullulan and amylopectin but, fail to cleave chemically identical bonds in glycogen (Manners, 1997).

Debranching enzyme involvement in starch biosynthesis is highly controversial causing much debate. Erlander (1958) speculated that amylopectin would be generated by debranching glycogen and that amylose would be formed by the debranching of amylopectin. Pan and Nelson (1984) proposed that starch branching and debranching enzymes work in concert to determine the fine structure of amylopectin and that the activity of branching and debranching enzymes must be precisely balanced in starch formation. This theory that starch synthases and debranching enzymes are solely responsible in determining amylopectin branching is backed up by work carried out on isoforms of branching enzymes (review by Smith *et al*., 1995).

Su1 (sugary1) mutants of cereals including maize and rice are defective in some aspect of starch granule biosynthesis, they accumulate phytoglycogen accompanied by significantly lower starch levels. Isoamylase and limit dextrinase activities are both reduced in the endosperms of allelic *sugary1* mutants, the isoamylase activity decrease is more highly pronounced than that of limit dextrinase (Kubo *et al*., 1999). The ratio of branching to debranching enzyme is markedly lower in mutants when compared to the wild type (Nakamura, 1996). *Su1* is part of a multigene family; its cDNA sequence specifies a polypeptide consisting of at least 742 amino acids (James *et al*., 1995). Amino acid sequence of *su1* establishes it as a member of the α-amylase family 13 of starch hydrolytic enzymes. Isolation of the *su1* gene by transposon tagging postulated it to encode an isoamylase, antibody analysis confirmed these classifications. Mutants

have an incomplete defective phenotype and are thus not entirely redundant, the *su1* phenotype is a consequence of a decrease in the amount of debranching enzyme activity, not an alteration of enzymatic properties. It is plausible that additional enzyme isoforms with similar functions are expressed during different stages of endosperm development (Ball, *et al*., 1996).

The water soluble polysaccharide (WSP) clearing model suggests that debranching enzymes eliminate soluble glucan from the stroma, thus removing substrates which compete for branching enzyme and starch synthase binding (Zeeman *et al*., 1998). The clearing model could account for the formation of phytoglycogen in mutants (Figure 1.3), it suggests that debranching activity plays an indirect role in starch biosynthesis by removing unattached soluble glucans from the stroma preventing random synthesis of glucan polymers by starch synthases and starch branching enzymes.

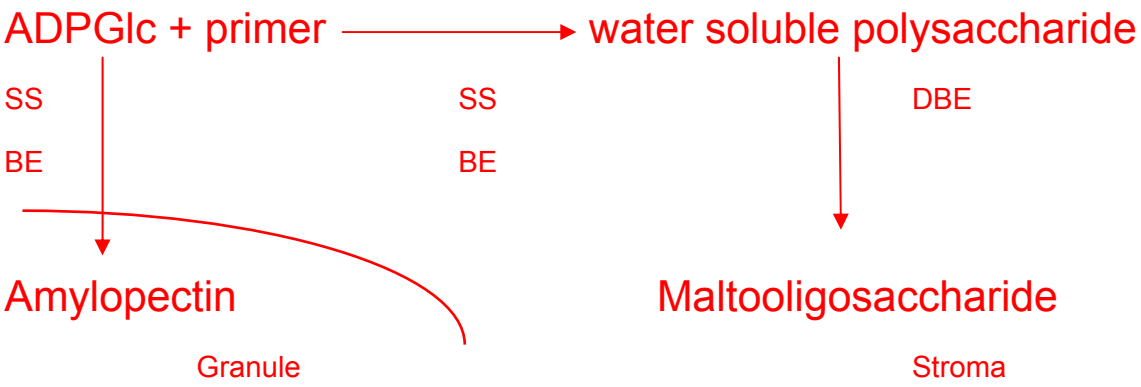

Figure 1.3: The WSP clearing model of starch biosynthesis in barley. SS: starch synthase, BE: branching enzyme, DBE: debranching enzyme. Diagram adapted from Myers *et al*., 2000.

The pre-amylopectin trimming model suggests that amylopectin biosynthesis consists of the formation of a 'pre-amylopectin', which is subsequently trimmed by debranching enzymes to mature amylopectin (Figure 1.4) (Myers *et al*., 2000). Debranching activity is postulated to be important in the removal of branches generated at the surface of the starch granule which would prevent crystallisation due to their incorrect positioning.

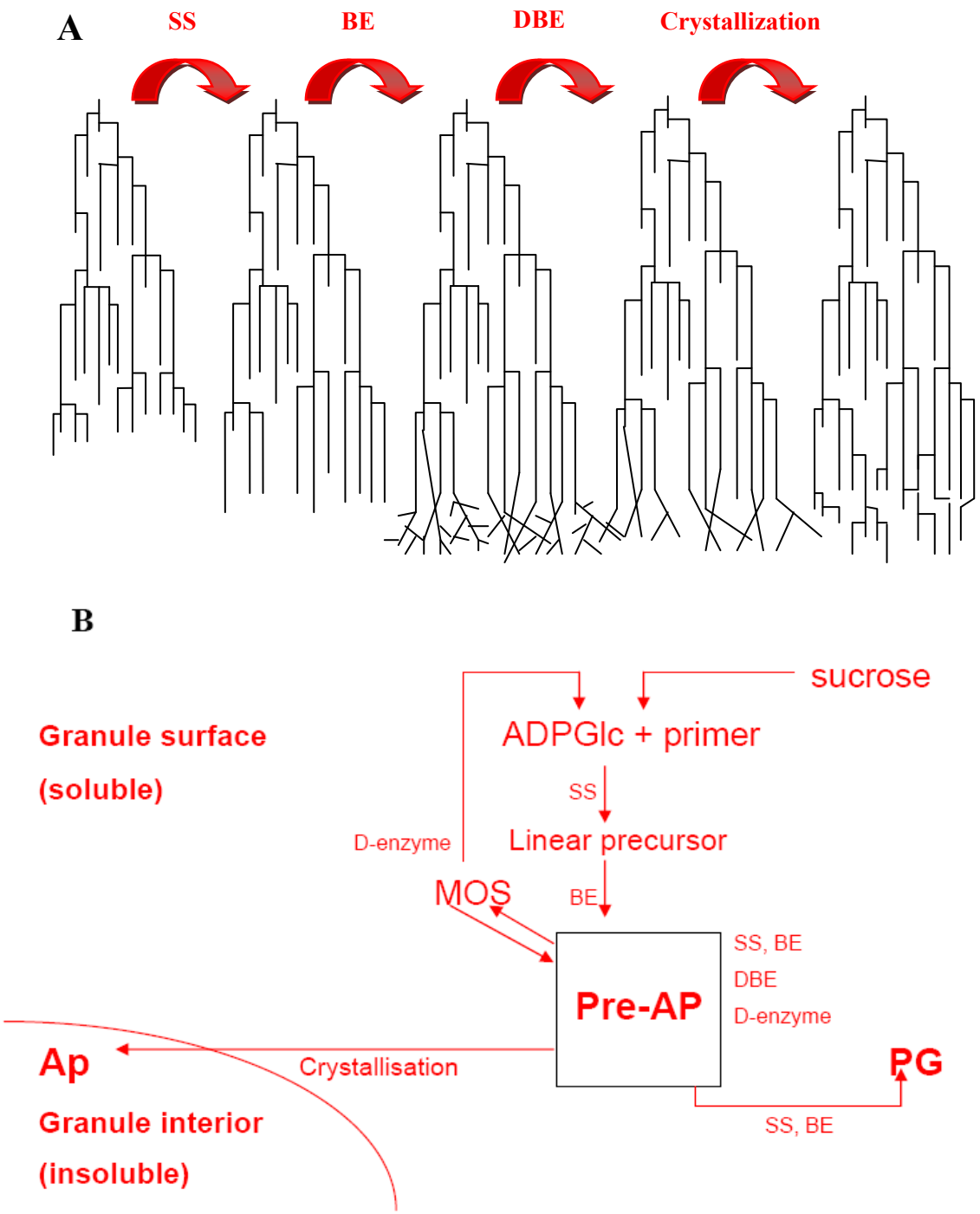

Figure 1.4: The pre-amylopectin trimming model in barley. SS: starch synthase, BE: branching enzyme, DBE: debranching enzyme. Diagram modified from Myers *et al*., 2000.

The evidence strongly suggests debranching of glucopolysaccharides to be a mandatory step of the normal starch biosynthetic pathway. The *su1* mutation affects not only the isoamylase level but, also pleiotropically affects limit dextrinase levels.

Since limit dextrinase protein levels were undetectable despite normal levels of mRNA transcript in the endosperm, the inhibition of limit dextrinase in su1 mutants is thought to occur at a post-transcriptional level (Kubo *et al*., 1999). It has been assumed that coordination of debranching enzymes, branching enzymes and starch synthase activities during starch synthesis is due to a physical association of enzymes within the amyloplasts, mutations at the *su1* locus could disrupt the complex through effects on debranching enzymes (James *et al*., 1995). In 2004 Stahl *et al*., demonstrated the importance of limit dextrinase and its proteinaceous inhibitor in the synthesis and breakdown of starch in barley, the limit dextrinase inhibitor gene was down regulated by antisense. Free limit dextrinase activity was enhanced in developing and germinating grains, unpredicted pleiotropic effects on α-amylase, β-amylase and starch synthase were also observed. These changes in enzyme activity were accompanied by reduced levels of total starch, reduced numbers of B type granules and reduced amylose to amylopectin ratios. Amylopectin chain length was also modified, there were fewer long chains (greater than 25 units) and enhanced levels of medium chains (between 10 and 15 units in length).

Starch phosphorylase (SP E.C.2.4.1.1) and plastidyl SP (referred to as PhoI) expression and activity correlate with a role in starch biosynthesis. Starch phosphorylase catalyses the reversible transfer of glucosyl units from Glucose-1-phosphate (Glc-1-P) to the non reducing end of α-1,4 linked glucan chains. The role of PhoI is unclear, it may be controlling the availability of malto-oligosaccharides required for amylose synthesis and acting in a clearing role complimentary to that of debranching enzymes, a number of studies have found that SP/PhoI gene expression and activity is correlated with starch biosynthesis (Van Berkel *et al*., 1991; Yu *et al*., 2001).

1.2.2. Starch degradation

A common feature of plant starches is that at some point they must be degraded (storage starches during germination). Starch granule structure must either be uniformly degraded from the outside or have built in entry points for hydrolytic enzymes.

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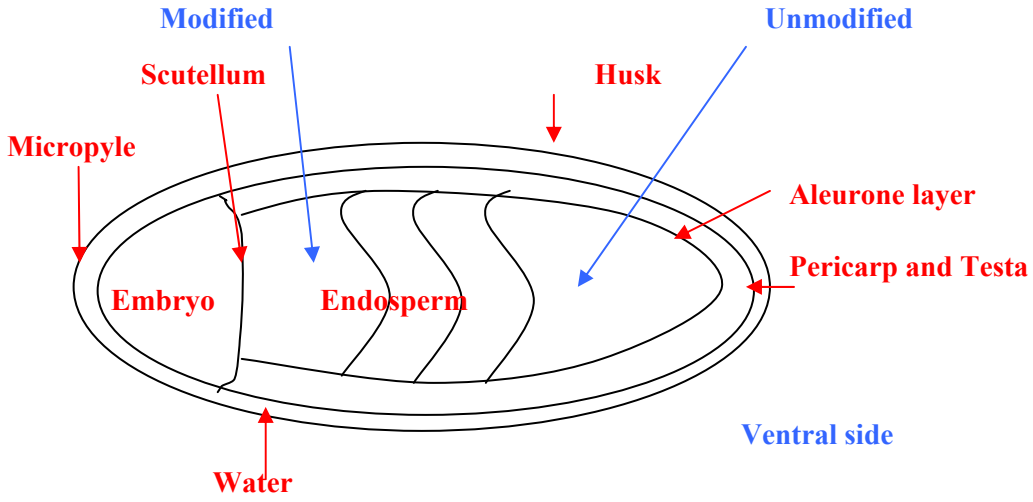
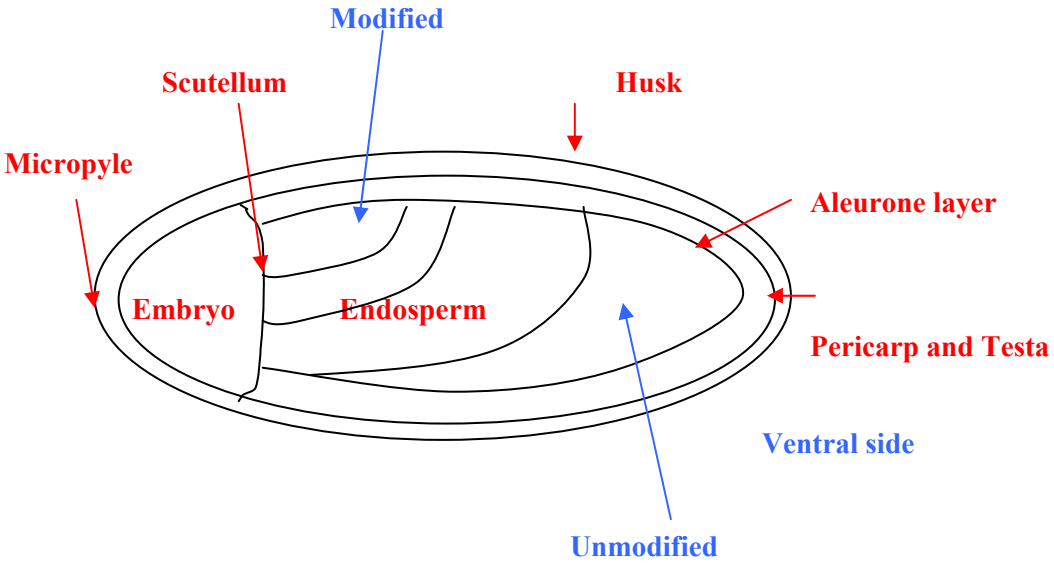
Starch granules in the endosperm are encased in a cell wall (which makes up ~10 % total material in starchy endosperm) made up of ~80 – 85 % β-glucan chains (500 to 20000 β-glucose molecules with β-1, 3 and β-1, 4 linkages) and ~15 – 20 % pentosan gums (Bamforth *et al*., 1983). In order for the enzymes secreted from the aleurone and scutellum to convert starch into sugars during barley grain germination and malting the glucan chains encasing the starch granules must be degraded (Bamforth and Martin, 1983; Woodward *et al*., 1982; Palmer *et al*., 1989; Palmer, 2006). There is little degradation of pentosan gums during malting however there is full degradation of the β-glucan gums. The conversion from a strong compact, non-porous matrix to a friable, well modified structure is based on the action of mainly β-glucanase, which randomly attacks β-1, 3 and β-1, 4 glucosidic linkages. Uniform modification is advantageous to the maltster as under such conditions the critical fermentation time can be reliably determined resulting in a greater conversion of starch to sugars. There are many differing viewpoints associated with hydrolytic enzymes tissue of origin and movement patterns within the kernel. Two conflicting models have been proposed to illustrate modification patterns (Figure 1.5). The Briggs model (Figure 1.5 a) (Briggs and MacDonald, 1983) speculates that the modification front progresses parallel to the scutellum layer from the proximal to the distal end of the grain while the Palmer model (Figure 1.5 b) (Palmer, 1983) suggests a pattern of modification progressing from the scutellum/aleurone junction on the dorsal side (O'Brian and Fowkes, 2004). Evidence shows that 42 to 70 h after steeping the protein matrix surrounding the starch granules remains intact, rapid digestion of the sub-aleurone layer can be observed between 70 and 100 h post steep, the central part of the endosperm remains undigested until after 100 h.

MacGregor, (1994 a) illustrated that during malting starch hydrolysis is restricted to the starch granules in the endosperm periphery. Good malting cultivars exhibit rapid and uniform breakdown patterns while poor malting varieties have slow, irregular endosperm breakdown despite showing similar levels of extractable enzyme activity (Brennan *et al*., 1997).



a. Briggs model – Modification front progresses parallel to the scuttellum layer from proximal to distal end.



b. Palmer model – Pattern of modification progresses from scuttellum/aleurone junction on dorsal side.

Figure 1.5: The Briggs and Palmer proposed models of starch endosperm modification. Water enters via the micropyle. Diagram adapted from O'Brian and Fowkes. 2004.

Starch degradation requires an initial hydrolytic attack by α-amylase on the intact starch granule, followed by the debranching of α-1, 6 glucosidic linkages to produce linear glucan chains which can then be degraded to glucosyl monomers. The main enzymes involved in starch degradation are α-amylase, β-amylase, limit dextrinase and α-glucosidase (Figure 1.6).

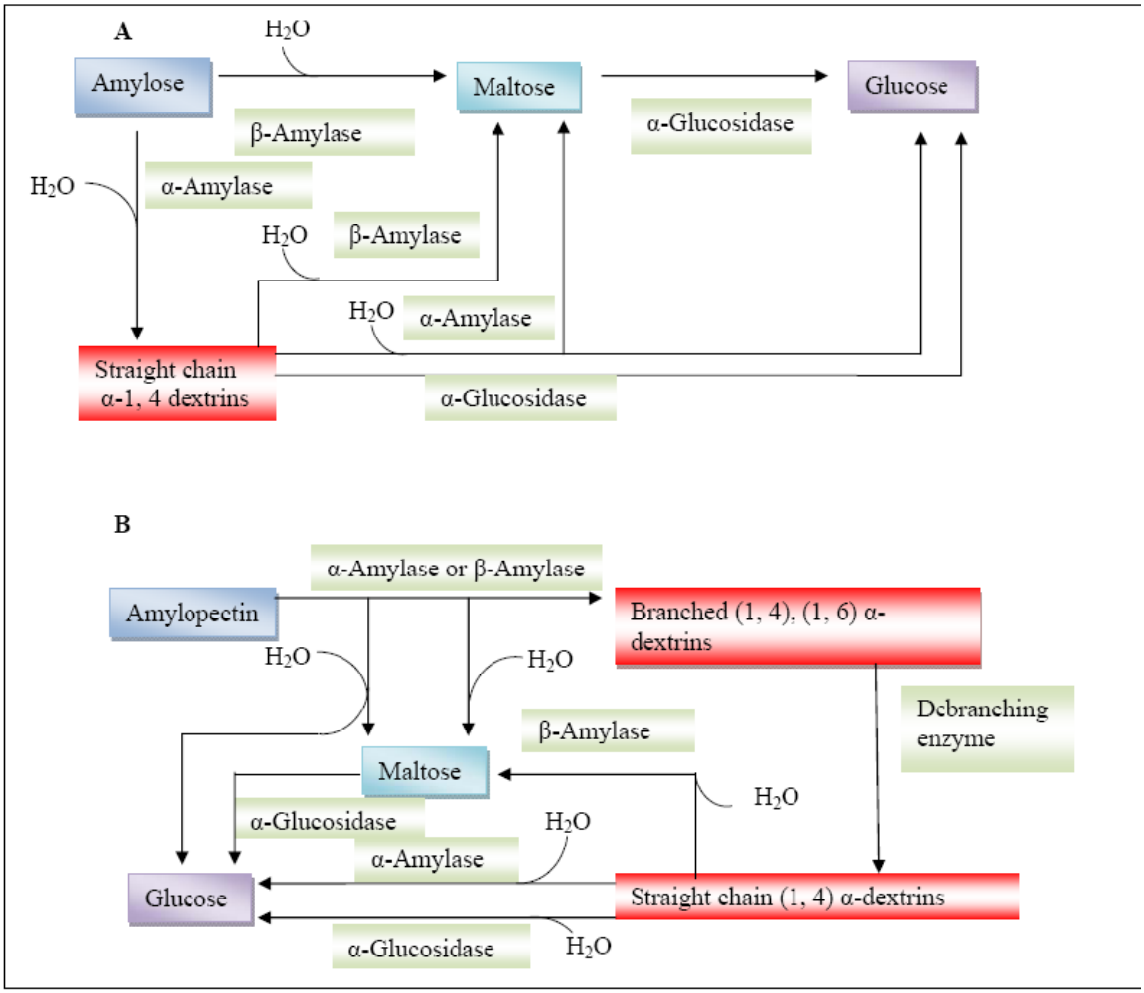


Figure 1.6: Enzymes involved in amylose hydrolysis (A) and amylopectin hydrolysis (B). Diagram adapted from Buchanan *et al*., 2001.

When the temperature of a slurry (malt flour and water) increases during mashing the starch granules change structurally, they absorb water, swell and eventually disrupt and solubilise forming a starchy gel (Atwell *et al*., 1988). This gelatinisation occurs at different temperatures depending on cereal type and granule size. Barley malt starch generally gelatinises between 56 and 62 °C (MacGregor *et al*., 1993; MacGregor *et al*., 1999), the smaller the granule size the higher the gelatinisation temperature, large starch granules normally gelatinise at 62 °C while small starch granules can require temperatures in excess of 75 °C (Hough et al., 1981). Gelatinisation caused by

mashing allows hydrolytic enzymes easy access to starch for degradation. α-Amylase action on gelatinised starch leads to the production of glucose, maltose, maltotriose and dextrins. β-amylase acts on gelatinised starch and dextrins to give only maltose (Brandam *et al*., 2003).

1.3. Barley hydrolytic enzymes

The efficiency of any one hydrolytic enzyme in a mash is influenced by the presence of others (Figure 1.6). β-Amylase and limit dextrinase work in concert (synergistically) to produce increased amounts of maltose in the mash liquor. It is commonly accepted that commercial malts contain sufficient levels of β-amylase but, less than optimal amounts of limit dextrinase (MacGregor *et al*., 1999). Addition of limit dextrinase in the mashes results in increased levels of fermentable sugars (MacGregor *et al*., 1999). Gelatinised starch is predominantly broken down by α and β-amylases to glucose, maltose and maltotriose as well as smaller quantities of longer α-1,4 linear maltodextrins and branched dextrins containing α-1,6 glucosidic bonds (Figure 1.7; Brandam, *et al*., 2003). Degradation of dextrins containing α-1,6 glucosidic bonds into glucose, maltose and maltotriose is facilitated by limit dextrinase acting in concert with α and β amylases (Figure 1.7).

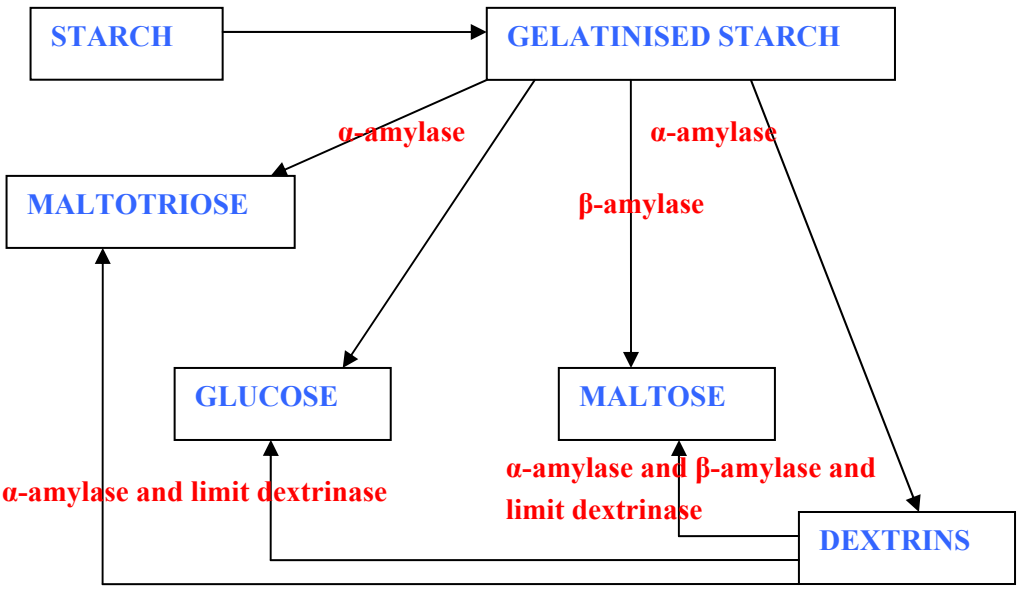


Figure 1.7: A model for the enzymatic hydrolysis of barley starch during mashing. Diagram adapted from Brandam *et al*., 2003.

1.3.1. Barley α-amylase

α-Amylase (1, 4-α-D-glucan glucanohydrolase, E. C. 3. 2. 1. 1) is an endo-acting enzyme, its action involves the essentially random cleavage of non terminal α-1,4 D-glucosidic linkages in both linear and branched components yielding an array of dextrins and oligosaccharides with C1-OH in α configuration (Wong *et al*., 2003 a). Barley malt contains two main α-amylase isoforms AMY1 and AMY2, they are readily distinguished by their isoelectric points: AMY1 has a low pI of 4.7 to 5.0 while AMY2 has a high pI of 5.9 to 6.1. AMY1 is synthesised during grain development (MacGregor, 1971) and is mainly associated with the outer pericarp tissue, AMY1 is virtually absent from the mature barley (MacGregor, 1977, 1983; MacGregor and Dushnicky, 1989). AMY1 and AMY2 are both synthesised *de novo* in the embryo and aleurone layer during germination, large proportions are secreted into the starchy endosperm (Briggs, 1998). Variance in the amount of α-amylase and the activity have been observed in different cultivars (Georg-Kraemer *et al*., 2001). AMY2 is the dominant form comprising 80 to 90 % of α-amylase activity. AMY1 and AMY2 vary in thermostability, AMY2 has a higher temperature tolerance but a lower temperature optimum. The BASI (bifunctional amylase subtilisin inhibitor) protein inhibits AMY2 (Mundy *et al*., 1983; Weselake *et al*., 1983; Nielsen *et al*., 2003).

Cereal α-amylases fall into two major gene classes: AMY A and AMY B. AMY A is divided into AMY1 and AMY2 subfamilies. AMY3 has been identified in barley, there is however a low copy number of the AMY B class in the barley genome. The AMY B (AMY3 subfamily) genes in barley are underrepresented when compared to other cereals (Huang *et al*., 1992).

Dormant and non dormant barley kernels produce α-amylase in response to the addition of GA_3 (at a much lower rate in dormant grain). The barley aleurone layer is 3 cells thick, Schuurink *et al*., 1997 revealed that all 3 aleurone layers showed cytodifferentiation of activated cell clusters in response to GA_3, only a small fraction of aleurone cells respond to GA_3 in dormant grain. Grain which exhibits no embryo dormancy, and thus appear to be of suitable malting quality, can show moderate aleurone dormancy.

1.3.2. Barley β-amylase

β-Amylase (1, 4-α-D-glucan maltohydrolase, E. C. 3. 2. 1. 2) is a member of family 14 according to IUBMB classification. It is a monomeric, exo-acting enzyme which catalyses the hydrolysis of α-1, 4 D glucosidic linkages in polysaccharides to remove successive β- maltose units from the non-reducing end of 1, 4 glucans such as starch and glycogen. β-Amylase cannot utilise raw starch, it hydrolyses the products of α-amylase activity, β-amylase cannot pass the α-1,6 branch points in amylopectin and is thus also dependent on limit dextrinase activity to facilitate complete production of fermentable sugars (Bamforth and Quain, 1989). Barley β-amylase has a molecular weight of 53 to 64 kDa and a considerable degree of amino acid sequence homology to other cereal β-amylases (as determined by cDNA sequence homology); there is a highly conserved glutamine residue (indicative of active site) (Wong, 2003 b; Ziegler, 1999).

β-Amylase has a multiplicity of charges and pI values which can partly be attributed to multiple gene expression. β-amylase isoforms of diploid barley are encoded by single loci on the homologous group 4 and 5 chromosomes. 3 of 4 main barley isoforms isolated from barley represent proteolytic modifications of a single primary gene product (Ziegler, 1999).

There are 3 endosperm specific β-amylase alleles (*Sd2H* – type A has high thermostability, *Sd1* – type B has intermediate thermostability and *Sd2L* – type C has low thermostability) each have co-dominant inheritance. The differences in thermostability and electrophoretic properties of β-amylase types can be attributed to 6 amino acid substitutions and to the proteolytic cleavage of the C-terminal during malting and mashing. The level of β-amylase activity is increased by a deletion in intron 3 of the structural gene for the *Sd2H* and *Sd1* alleles resulting in increased expression. Differing characteristics of allelic forms of β-amylase have an effect on the fermentability of barley varieties.

β-Amylase exists in barley in ‘bound’, ‘free’ and ‘latent’ forms. The barley β-amylase enzyme lacks an N-terminal targeting signal sequence suggesting that it is synthesised as a mature protein in the cytosol and deposited in a bound form on the starch granule

surface when the endosperm cells lose their compartmental integrity. As β-amylase is a component of the starch protein matrix it prevents premature α-amylase attack.

'Bound' β-amylase occurs as a result of aggregation with itself and other seed proteins, post-translational modification (proteolysis and disulfide bond reduction) yields a 'free' active form which increases considerably during grain germination.

The 'bound' form makes up 50 % of total enzyme in mature grains. 'Free' β-amylase is water soluble, the 'bound' form is extractible in reducing conditions (i.e. being treated with thiol containing agents) or by proteolytic agents (Sandegren *et al*., 1950; Buttimer *et al*., 1998), the 'latent' form is extractable in detergent. β-amylase release and activation can be indirectly related to gibberellic acid, as it has hormonal control over the release, secretion and synthesis of proteases which have reportedly mediated enzyme release (MacGregor *et al*., 1971). Bound β-amylase is not completely redundant but, activity is significantly reduced due to the inability of the substrates to bind at the active site (Sopanen and Lauriere, 1989).

The main fermentable sugar produced during malting and mashing is maltose, which comprises 60 % of fermentable sugars present in wort, β-amylase is considered central in the determining wort fermentability as it hydrolyses oligoglucans with a degree of polymerisation of 5 to 6, while α-amylase is active on glucans with a degree of polymerisation of over 7. High β-amylase activity was observed in barley during days 2 and 3 germination (Georg-Kraemer *et al*., 2001), this has been attributed to the proteolysis and reduction of the 'bound' enzyme. β-Amylase contributes to starch granule hydrolysis by degrading solubilised intermediates released by α-amylase activity (Ziegler, 1999).

β-amylases contain a conserved cysteine residue, its modification by several sulfhydryl group oxidising reagents causes a decrease in enzyme activity. The attachment of bulky alkylating agents during chemical modification introduces steric stresses which cause structural distortion, prevent substrates from productively binding. β-amylase is also susceptible to inactivation by Ca^{2+} chelating agents. α-Cyclodextrin is a competitive inhibitor of β-amylase (Wong *et al.*, 2003 b). Protease inhibitors such as leupeptin and antipain were found to inhibit the release of bound β-amylase.

Yin *et al*., 2002 found significant differences in β-amylase activities between different cultivars and that different grains at differing positions on a spike showed significant variation. These relative differences along a spike also varied between cultivars. It was found that β-amylase activity was highest at the top of a spike and lowest at the bottom.

1.3.3. Limit dextrinase

In 1931 a debranching enzyme was recognised in yeast by Nishimura by increased iodine staining of amylopectin, the enzyme was called amylosynthease. This enzyme activity was subsequently found in potato tuber and the rice endosperm (Minagawa, 1932), it was later recognised that amylosynthease did not synthesise starch but, debranched amylopectin to produce linear glucans (Maruo and Kobayashi, 1949, 1951). Debranching enzyme, labelled R-enzyme was found in pea embryos and pullulanase was discovered in both plants and bacteria (Hobson *et al*., 1951). R-enzyme, amylosynthease and limit dextrinase were then found to be the same enzyme due to their substrate specificities (Peat *et al*., 1952; Nikuni, 1969). Limit dextrinase was the chosen nomenclature due to its relation to the natural substrate (α-limit dextrins) (Hizukuri, 1986; Kainuma, 1988).

In 1978 Maeda *et al.* found α and β-amylase activities to be at their highest in barley seeds following 6 d germination in the dark, debranching activity appeared a little later in the course of germination, this was later found to be limit dextrinase. Limit dextrinase (α-1, 6 glucanohydrolase, E. C. 3.2.1.142) is synthesised *de novo* during germination under the influence of gibberellic acid, it plays a complementary role to those of α and β-amylases.

Limit dextrinase selectively catalyses the slow hydrolysis of α-1, 6 branch points in amylopectin, pullulan and α-limit dextrins. The major role of limit dextrinase is the hydrolysis of starch degradation products during plant germination, limit dextrinase has also been implicated in the starch biosynthetic pathway (as described in section 1.2.1).

High levels of limit dextrinase in malt will increase the conversion of branched dextrins in the mash to linear oligosaccharides. Limit dextrinase activity requirements vary

between brewer and distiller, brewers require a certain amount of limit dextrinase activity to ensure an adequate supply of fermentable sugars are produced during mashing for yeast nutrition with sufficient dextrins remaining in the product to contribute to taste, texture and body. Distillers prefer complete dextrin degradation to increase fermentability and overall spirit yield (Bryce, 2003).

The barley limit dextrinase gene has been isolated and mapped to the long arm of chromosome 4 (Burton *et al*., 1999). Amino acid sequence analysis showed barley limit dextrinase to belong to the α-amylase family 13 of hydrolytic enzymes with a characteristic $(\beta/\alpha)_8$ barrel catalytic domain (Svensson, 1994). Burton obtained mRNA encoding barley limit dextrinase from overlapping cDNA clones from PCR amplification. The open reading frame encodes a putative transit peptide of 78 amino acid residues and a mature polypeptide of 884 amino acid residues. The calculated molecular weight of barley limit dextrinase is 97.4 kDa compared with an obtained weight of 105 kDa. The reported pI value of pure limit dextrinase ranges from 4.2 to 4.6 (Sissons *et al*., 1992; MacGregor *et al*., 1994 b; Kristenson *et al*., 1998). Molecular weight discrepancies are thought to be due to glycosylation of the enzyme, there are 7 potential N-glycosylation sites in the deduced amino acid sequence.

Limit dextrinase mRNA production is enhanced in the aleurone layer when treated with gibberellic acid and is also present in germinating barley grains. When germinating, barley grains treated with abscisic acid, an antagonist of gibberellic acid, limit dextrinase mRNA induction is abolished. The mechanism of limit dextrinase mobilization into the barley endosperm remains to be established (Bryce, 2003).

During development low levels of limit dextrinase mRNA are detected in the barley grain up to 20 d post anthesis, these low mRNA levels are accompanied by limit dextrinase activity within the grain, after 20 d post anthesis both mRNA and activity decline rapidly. Limit dextrinase mRNA expression patterns correspond closely with the timing of starch synthesis (M^{c}Leod and Duffus, 1988). Starch synthesis occurs in the amyloplast and limit dextrinase contains a presequence typical of transit peptides that target nascent polypeptides to such organelles.

Limit dextrinase is present in barley in a ‘free’ and ‘bound’ form. The ‘free’ limit dextrinase is extractable in buffer alone while the extraction of ’bound’ limit dextrinase requires proteolytic or reducing agents. In 1993 Longstaff and Bryce postulated that barley limit dextrinase is present in a bound inactive form following the initiation of germination. After 5 to 6 d germination the ratio of bound enzyme decreased while levels of free enzyme activity increased. Virtually all limit dextrinase was free following 9 d germination. Two hypotheses have been proposed for the apparent release of limit dextrinase *in vitro*. The first suggests that reducing ends such as L-*cys* activate the limit dextrinase by cleaving disulphide bonds (Yamada, 1981). This theory implies that limit dextrinase is a sulfhydryl enzyme, which requires free sulphydryl groups for activity. Sulphydryl compounds stimulate the conversion of bound limit dextrinase to its free form, perhaps because they activate malt thiol endoproteases or release limit dextrinase from inhibitors. The second hypothesis suggests a sulphydryl proteinase(s) is activated by reducing agents through the cleavage of disulphide bonds or is kept active via retention of disulphide bond formation. Longstaff and Bryce (1993) support theories that the bound form of barley limit dextrinase is released *in vitro* by the action of cysteine proteases activated by thiols.

Proteinaceous inhibitors have been found for barley limit dextrinase. Limit dextrinase inhibitor (LDI) is encoded for by a small multigene family, the LDI gene is expressed 2 to 4 weeks post anthesis in the endosperm and is active from 4 weeks post anthesis (only in the starch endosperm, not in the aleurone or embryo) (Stahl *et al*., 2004). Inhibitor levels are at their highest post anthesis and are found to decrease as germination progresses, LDI is broken down during germination (MacGregor *et al*., 2000). Inhibitors have been separated on the basis of their apparent isoelectric points and have been thus designated as high (pI 7.2) and low (pI 6.7) pI inhibitors (MacGregor *et al*., 1994 c). These inhibitors appear to undergo degradation during malting, sufficient levels remain to inhibit a high proportion of limit dextrinase activity. Limit dextrinase inhibitors share sequence homology with barley α-amylase trypsin inhibitor, despite being incapable of inhibiting both trypsin and α-amylase activity. Both inhibitors were heterogeneous with ‘ragged’ carboxyl ends. Molecular models showed 9 cysteine residues in the molecule, 8 of which are likely to be involved in intramolecular disulphide bonds and 1 free sulfhydryl group. The low pI inhibitor is the major component group, with a molecular mass of 12828 Da, it contains a gutathione residue probably bound to the sulfhydryl group. The high pI inhibitor has 2 components of molecular masses 12686 and 12744 Da, these contain cysteine residues bound to sulfhydryl groups. The two limit dextrinase inhibitors are encoded by the same gene but, have undergone different post transcriptional modifications. 3-Dimensional structure analysis revealed serine, valine and arginine residues which may be

intimately involved in enzyme-inhibitor interactions, the replacement of these residues with any other amino acid through genetic engineering results in a diminished ability to interact with limit dextrinase. The stoichiometry of the complex formed by barley limit dextrinase and its low pI endogenous inhibitor is a molar ratio of 1:1. The inhibitor complexes with and inhibits 7.5 times its weight as limit dextrinase is 7.5 times bigger, this ratio remains unaltered in excesses of either enzyme or inhibitor (MacGregor *et al*., 2002).

Cyclodextrins inhibit barley limit dextrinase, with β-cyclodextrin having a more detrimental inhibitory effect than its counterparts α-cyclodextrin and γ-cyclodextrin. Cyclodextrins are competitive inhibitors of barley limit dextrinase, as of several amylolytic enzymes, and thus bind at an active site (Kristensen *et al*., 1998).

1.4. Nitrogen

During malting barley storage proteins within the endosperm must be degraded sufficiently by proteinases to provide amino acids and peptides for yeast nutrition during fermentation. Protein degradation during mashing can also promote colloidal stability in beer. Nitrogen levels are a key quality parameter, protein degradation is critical in obtaining good quality malt (Lewis *et al*., 1992). The complete degradation of all barley proteins is undesirable as too little protein in the beer will result in poor foam stability and mouth feel.

The majority of proteins in the insoluble fraction prior to proteolysis are storage proteins, mainly hordeins, but various albumins and globulins are also likely to be degraded during malting (Jones 2005). The soluble protein fraction following proteolysis consists of a mixture of amino acids, peptides and dissolved proteins. At least 40 different endoproteinases have been detected in green malt using 2-dimensional isoelectric focusing and polyacrylamide gel electrophoresis (Zhang *et al*., 1995), multiple enzymes belonging to each of the 4 protease classes (aspartic, serine, cysteine and metalloproteases) were identified in the green malt. There is little or no inactivation of proteases during kilning (Jones *et al*., 2000) or the protein rest phase of mashing (Jones *et al*., 2002) so all have considerable opportunity to hydrolyse barley proteins thus providing sufficient nutrients for yeast.

1.5. Fermentable sugars

Starch hydrolysis to simple sugars during germination is the most fundamental process in brewing (Evans *et al*., 2005) In fermentation the yeast cell gains energy from sugars which it converts to ethanol and carbon dioxide, it only has the ability to utilise simple sugars. Despite being subjected to a variety of carbohydrates yeast cells generally consume the monosaccharides glucose and fructose first (Verstrepen *et al*., 2004).

Sucrose is a disaccharide which is broken down to glucose and fructose by extracellular invertase, removal of sucrose from the wort generally occurs within 3 to 12 h depending on invertase activity of yeast species. Once these carbohydrates are utilised the yeast will then transport maltose, the most abundant fermentable sugar of wort, into the cell whereby it is hydrolysed to glucose. It is the transport of maltose in the yeast cell as opposed to its hydrolysis which is the rate limiting step determining fermentation performance (Menese *et al*., 2002). Maltose metabolism in *Saccharomyces cerevisiae* requires the presence of at least one of five independent *MAL* loci: *MAL 1, MAL 2, MAL 3, MAL 4* and *MAL 6*. Each locus is made up of three genes each encoding intracellular maltase, the transport protein for maltose and a positive regulatory protein. Maltose is transported unchanged into the cell with the help of a specific transmembrane transporter where it is hydrolysed by maltase into two glucose units which are then channelled through the glycolytic pathway. Maltose assimilation and metabolism is controlled by three regulatory mechanisms: induction, glucose repression and glucose inactivation (Novak *et al*., 2004). Lastly most yeast strains will utilise maltotriose. In standard wort maltotriose is the second most abundant sugar; its efficient fermentation is a desired property of brewing strains of *Saccharomyces cerevisiae*. The presence of residual maltotriose in beer cannot be attributed to a genetic or physiological inability of yeast cells to metabolise it but rather to a low affinity for maltotriose uptake by maltose permease (Day *et al*., 2002).

The presence of glucose and sucrose causes repression of glucanogenesis, the glyoxylate cycle and the repression of uptake of less preferable carbohydrates. Glucose and sucrose can also exhibit unexpected hormone-like effects including the activation of cellular growth, the mobilisation of storage compounds and the diminution of cellular stress resistance. Effects brought about by the presence of glucose and sucrose in the wort can lead to several yeast related problems in an industrial context including slow or incomplete fermentation, ‘off’ flavours and the poor maintenance of yeast vitality (Verstrepen *et al*., 2004).

1.5.1. Sugar regulation of gene expression and enzyme activity

In plants the sugar status often modulates and coordinates internal regulators and environmental cues that govern growth and development (Koch *et al*., 1996). Recent progress has begun to outline the mechanisms of sugar sensing and signalling in plants.

Hexokinase is a glucose sensor, it acts as the primary sugar sensor in plants modulating gene expression of participants in various hormone dependent signalling pathways (Sheen *et al*., 1999; Smeekens *et al*., 2000).

In general sugars favour the expression of enzymes in connection with starch biosynthesis and the storage of reserves during grain development (Koch, 1996), increased sugar concentrations can lead to an increase in Starch biosynthetic enzyme expression (i.e. ADP-Glc pyrophosphatase, invertase and sucrose synthase), these proteins related to starch biosynthesis appear to be positively regulated by sugars (Müller-Röber *et al*., 1990).

Increased sugar concentration generally results in a decrease of hydrolytic enzyme expression during germination, such as the repression of α-amylase induction by glucose, fructose or sucrose (converted to glucose and fructose by invertase prior to cell uptake) in *Oryza sativa* (Ho *et al*., 2001). Sugar induced down regulation involves control at both transcriptional and mRNA stability levels as occurs for α-amylase in germinating barley embryos (Loreti *et al*., 2000) and germinating rice (*Oryza sativa cv Tainin 5*) embryos and cell suspensions (Yu *et al*., 1991, 1996).

Under conditions of sugar deprivation substantial biochemical and physiological changes take place, such as reduction in cell growth, decreases in cellular starch and sugar levels as well as decreased respiration and metabolic rates. As barley grain germination occurs, the aleurone layer becomes progressively less capable of sustaining increased α-amylase production in response to GA_3. The decline in α-amylase production is linked to the developing embryo which modulates the response to GA_3. Sugars repress α-amylase formation in the barley grain by reducing the levels of endogenously produced gibberellins (Smith *et al*., 1980).

Extended extractions of barley malt was carried out by MacGregor in 2002, when pullulan type substrates were used in the assay the accumulation of starch degradation products initially activated the malt limit dextrinase, higher levels of sugars were found to inhibit enzyme activity. This activation of malt limit dextrinase, caused by increased maltose concentrations, was not observed when a more natural substrate was used in the assay. Maltodextrins were reported to alter the mode of action of limit dextrinase, causing a more rapid decrease in viscosity or causing a greater solubilisation of dye linked pullulan fragments per cleavage event, transglycosylation was inhibited by unlabelled maltotriose and accounted for the apparent activation of limit dextrinase by maltodextrins.

Low molecular weight oligosaccharides such as maltose, maltotriose and maltosaccharides were found to have an inhibitory affect on the activity of rice limit dextrinase (M^{c}Dougall *et al*., 2004).

1.6. Post-translational regulation of enzyme activity

1.6.1. Redox modulation of starch metabolic enzymes

Redox cascades in the chloroplast and mitochondria of plant cells generate signals which participate in and regulate gene expression and translation of enzymes (Foyer *et al*., 2003). Molecular oxygen in electron transport processes generate powerful redox signals including superoxide, hydrogen peroxide and singlet oxygen. Evidence suggests the involvement of the plant cell redox buffers ascorbate and glutathione in redox signal transduction. In wheat ascorbate and glutathione redox enzyme activities are high before the start of drying and maturation after which they decrease (DeGara *et al*., 2003). Ascorbate and glutathione pairs and the sulfhydryl to disulfide transition in proteins are parameters which are tightly related to wheat grain maturation. Reactive oxygen species are involved in redox regulation by their capacity to reversibly oxidise cysteine residues. Controlled production of reactive oxygen species (ROS) acts as a secondary messenger alongside mediators such as calcium in a plant's response to hormone signalling, allowing cells to modulate different responses to external stimuli (Pei *et al*., 2000).

In the barley aleurone layer, gibberellic acid is perceived at the membrane and induces the synthesis and secretion of α-amylase (Dominguez *et al*., 1999). At the same time intense glucanogenesis of lipid reserves generates reactive oxygen species hydrogen peroxide as a by-product (Maya-Ampudia *et al*., 2006) shortly after this event the cells undergo programmed cell death (Palma and Kermode, 2003). Redox changes occur in membrane proteins after gibberellic acid signalling in the aleurone layer (17 constitutive proteins were reduced in response to gibberellic acid while 5 were oxidized). In the presence of gibberellic acid the aleurone underwent programmed cell death between 18 and 48 h, during the first 24 h the ability of aleurone cells to metabolize hydrogen peroxide was increased. Aleurone cell death is reportedly the final step in a developmental process resulting in a viable seedling (Betke *et al*., 1999).

Despite having a common origin only the aleurone cells remain alive at maturation while the endosperm cells are dead (Fincher, 1989). In the later stages in barley grain development the aleurone cells secrete malic acid into the endosperm (Macnicol *et al*., 1992). The barley endosperm actively maintains pH at between 4.9 and 5 (Mikola *et al*., 1980), this acidic pH often enhances hydrolytic enzyme activity as observed for α-amylase (Fischer *et al*., 1951), cysteine proteases (Koehler *et al*., 1990) and limit dextrinase (M^{c}Cafferty *et al*., 2004). Low pH was found to elevate the aleurone synthesis and secretion of gibberellic acid and hence α-amylase abundance (Sinjorgo *et al*., 1993). Endosperm acidification by the aleurone layer obviously plays an important part in the degradation and mobilisation of nutrient reserves.

1.6.2. Thioredoxin

Thioredoxins are protein disulfide reductases, they have molecular masses of ~ 12 kDa (Homgren, 1985). The structure of thioredoxin is highly conserved among species, it consists of a central 5-stranded β sheet surrounded by 4 α-helices in a βαβαβαββα topology (Holmgren *et al*., 1985; Katti *et al*., 1990; Menchise *et al.*, 2001). The reduced dithiol form of thioredoxin can modulate the activity of a variety of target proteins by reducing their disulfide bonds. Plants contain several forms of thioredoxin which differ in sub cellular localization and target proteins (Schürmann *et al*., 2000; Baumann *et al*., 2002).

Thioredoxin h has been found to have an important influence on barley grain germination (Yano *et al*., 2001; Marx *et al*., 2003). The redox activity of thioredoxin h involves 2 cysteine residues in the active site motif $WC_NG(P)PC_C$.

In an oxidised state the 2 cysteines form an intramolecular disulfide, reducing equivalents are obtained from NADPH via thioredoxin reductase (Arnėr *et al*., 2000). In reduced state, the surface exposed cysteine at the N-terminal side of the motif (C_N) attacks the target protein's disulfide bonds forming an intermolecular disulfide intermediate, subsequent attack by the buried C-terminal cysteine (C_C) results in the release of a reduced target protein and an oxidized thioredoxin (Figure 1.7) (Kallis *et al*., 1980).

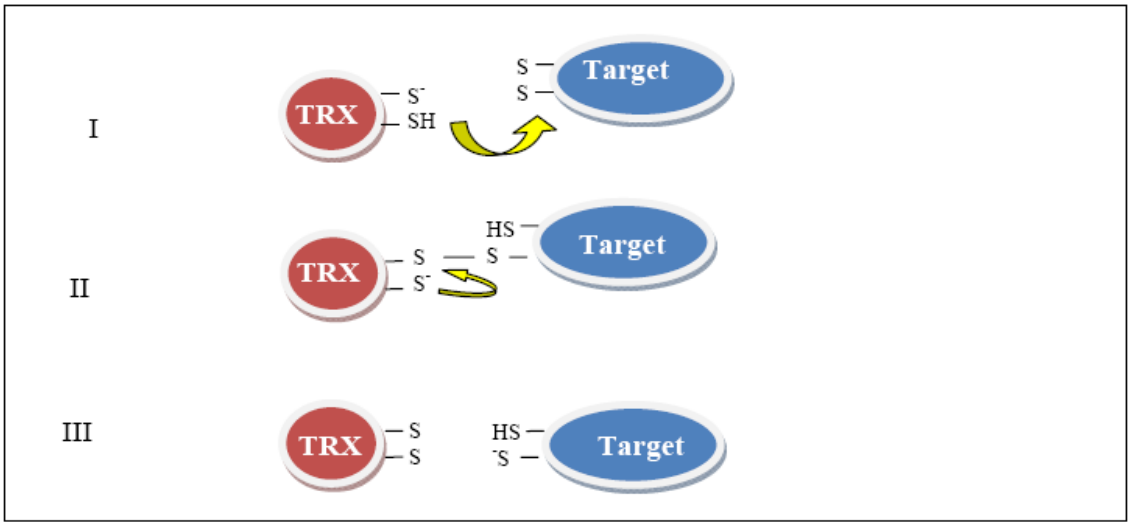


Figure 1.8: Mechanisms of reduced thioredoxin activity. Diagram adapted from Maeda, *et al*., 2006

In the endosperm of mature wheat seeds 23 proteinaceous thioredoxin targets were identified by reducing tissue extracts with thioredoxin, treating with monobromobimane (a fluorescent probe which labels sulfhydryl groups), isolating labelled proteins on 2 dimensional electrophoresis or SDS-PAGE and identifying these proteins using amino acid sequencing. Of the 24 targets identified 12 corresponded to metabolism, 3 to protein storage, 3 to oxidative stress response, 2 to protein degradation, 1 to protein assembly and folding and 2 of which were unknown (Wong *et al*., 2003 c).

Proteomic analysis revealed 2 thioredoxin h isoforms in barley grains which are 51 % identical in sequence, isoforms displayed higher similarity to thioredoxin of other cereal

sources (Trxh1 was 71 % identical to rice Trxh, Trxh2 shared 90 % identity to wheat TrxTa) (Maeda *et* al., 2003). HvTrx1 was observed in the endosperm, aleurone layer and embryo (while remaining high in the embryo levels decreased in the aleurone layer during germination). HvTrx2 is present mainly in the embryo, it decreased in abundance during germination. The 2 barley thioredoxin h isoforms differ in distribution and kinetic properties, it can be assumed that they have different physiological roles.

In barley thioredoxin h is converted from an oxidised to a partially reduced state one day after imbibition, later in germination levels of thioredoxin h decreased (Besse *et al*., 1997). When thioredoxin h was over expressed in the starchy endosperm of barley it was found to communicate with the embryo and aleurone, accelerating germination and α-amylase appearance, it was also speculated that thioredoxin h enhanced the synthesis of gibberellic acid in the embryo (Wong *et al*., 2002). Thioredoxin h appears to provide a redox-linked mechanism whereby the embryo and aleurone can sense the biochemical status of the endosperm and adjust their activities accordingly.

When active wheat thioredoxin h gene was fused to an endosperm specific B1-hordein promoter and over expressed in transgenic barley grains a 4-fold increase in limit dextrinase activity was observed (Cho *et al*., 1999). The proposed mechanism suggests that thioredoxin reduced by NADPH via NTR reduces the disulphide bonds present in the inhibitor allowing the targeted limit dextrinase to become active. Results however established that enhanced limit dextrinase activity was not brought about by inhibitor inactivation but, by a mechanism yet to be established. It is possible that thioredoxin acts by increasing *de novo* limit dextrinase synthesis or by preventing the limit dextrinase from binding to the starchy endosperm.

Thioredoxin h (reduced either enzymatically with NADP-thioredoxin reductase or chemically with DTT) was found to reduce α-amylase and trypsin inhibitors from several sources (including the wheat α-amylase inhibitor and the maize trypsin inhibitor) (Kobrehel *et al*., 1991). These results suggest that in the absence of compartmental barriers, reduction can take place within the cell and it is plausible that thioredoxin could control cellular activity of α-amylase or protease enzymes by changing the redox status of their corresponding inhibitor proteins.

Barley α-amylase/subtilisin inhibitor (BASI) is a thioredoxin h target (Jiao *et al*., 1993). Thioredoxin target protein screening in barley seed extracts was carried out, results showed that C144-C148 of the α-amylase/subtilisin inhibitor (BASI) was reduced by thioredoxin h, this disulfide was preferentially reduced compared to the C43-C90 disulfide in BASI (Maeda *et al*., 2003; Maeda *et al*., 2004; Maeda *et al*., 2005).

During cereal grain germination dramatic physiological changes occur constituting critical transitions in the life cycle of the plant. Carbon and nitrogen sources are provided for the seeding by starch and nitrogen mobilization in the endosperm prior to the initiation of photosynthesis. Evidence suggests that thioredoxin promotes carbon and nitrogen mobilisation in early seedling development. The major storage proteins in wheat (gliadins and glutenins) are specifically reduced by thioredoxin (Kobrehel *et al*., 1992; Wong *et al*., 1993). Gliadin and glutenin reduction takes place early in grain germination reaching a peak 2 to 3 d after imbibition (Kobrehel *et al*., 1992). At the same time the endosperm fraction contains the enzymes necessary to reduce NADP by the oxidative pentose phosphate pathway (hexokinase, glucose-6 phosphate dehydrogenase, 6-phosphoglucanate dehydrogenase). The NADPH formed can reduce indigenous thioredoxin h via NTR in the endosperm (Lozano *et al*., 1996).

The role of thioredoxin in wheat starch metabolism, protein breakdown and oxidative stress has been established (see Figs. 1.9 to 1.11).

In regard to starch (Figure 1.9) thioredoxin regulates breakdown directly via inactivating specific inhibitor proteins (the α-amylase and limit dextrinase inhibitor proteins) and enhancing limit dextrinase synthesis and downstream via ADP-glucose pyrophosphorylase. Thioredoxin also acts in the conversion of fructose 1,6-bisphosphate and fructose 6-phosphate and at other points in the glycolytic pathway (activation of aldolase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, enolase and PPDK) (Wong *et al*., 2003 c).

Thioredoxin also has a role in regulating protein degradation (Figure 1.10). Thioredoxin appears to facilitate the degradation of storage proteins directly via reductive unfolding (PDI) and the activation of proteases such as thiocalsin and serpin.

Enzymes such as alanine aminotransferase and NAD-malate dehydrogenase are also activated by thioredoxin (Wong *et al*., 2003 c).

There is also a role for thioredoxin in the plant response to oxidative stress (Figure 1.11). Thioredoxin acts as an electron donor peroxiredoxin and facilitates the removal of hydrogen peroxide via the regulation of GSH-dependent dehydroascorbate reductase and ascorbate peroxidase (Wong *et al*., 2003 c).

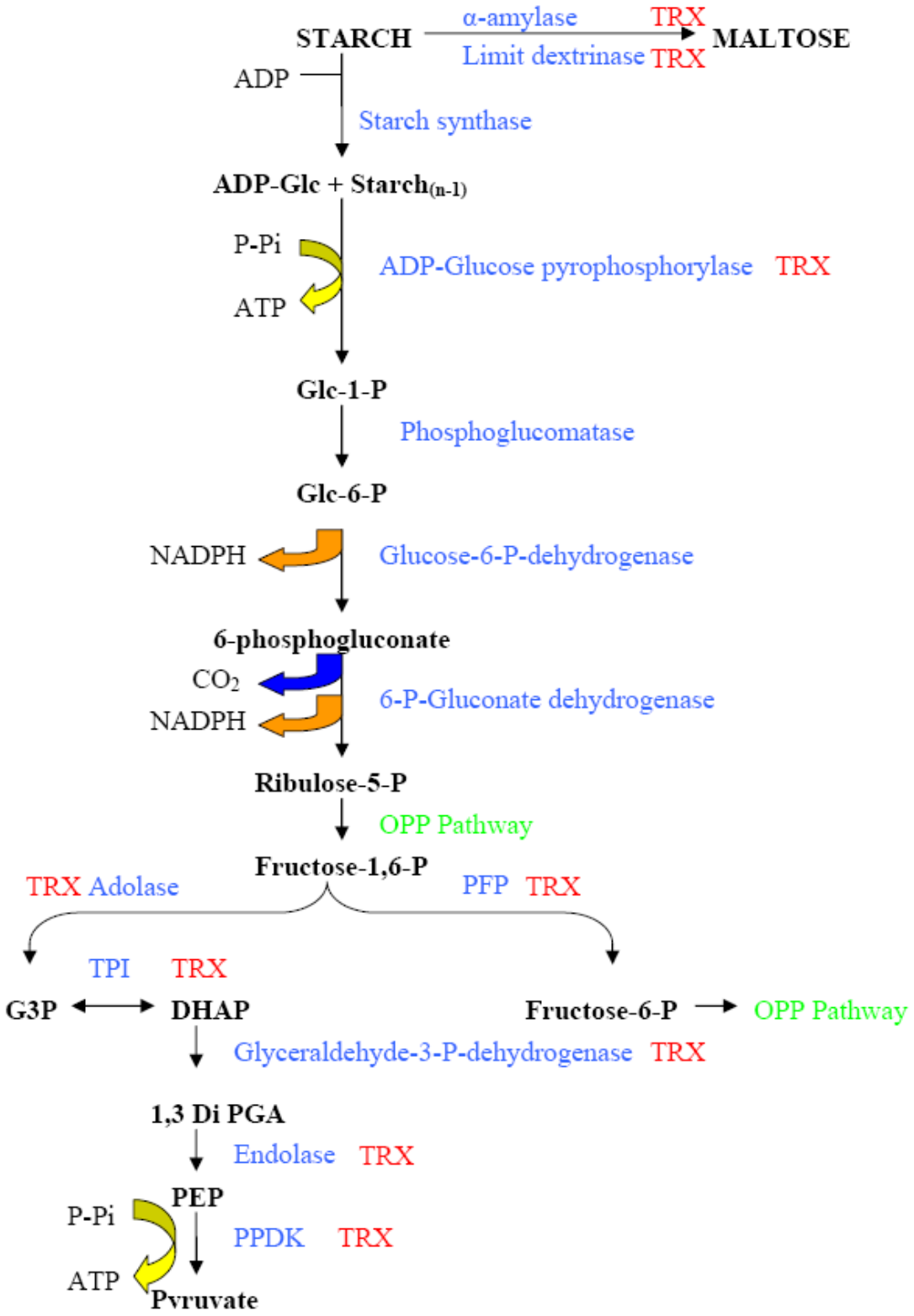


Figure 1.9: Role of thioredoxin h in starch breakdown in the wheat endosperm. TRX in the diagram appears alongside the enzymes which are known thioredoxin targets. Diagram adapted from Wong et al., 2003 c.

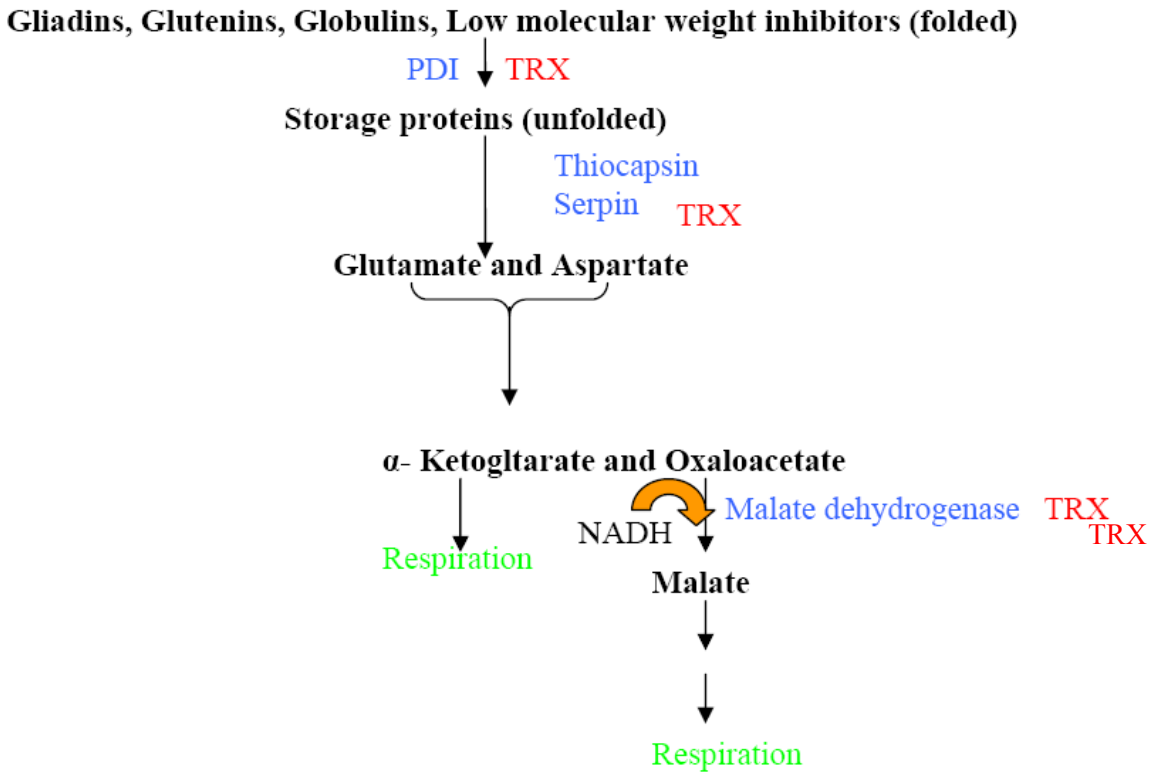


Figure 1.10: Role of thioredoxin h in protein breakdown in wheat endosperm. TRX in the diagram appears alongside the enzymes which are known thioredoxin targets. Diagram adapted from Wong *et al*., 2003 c.

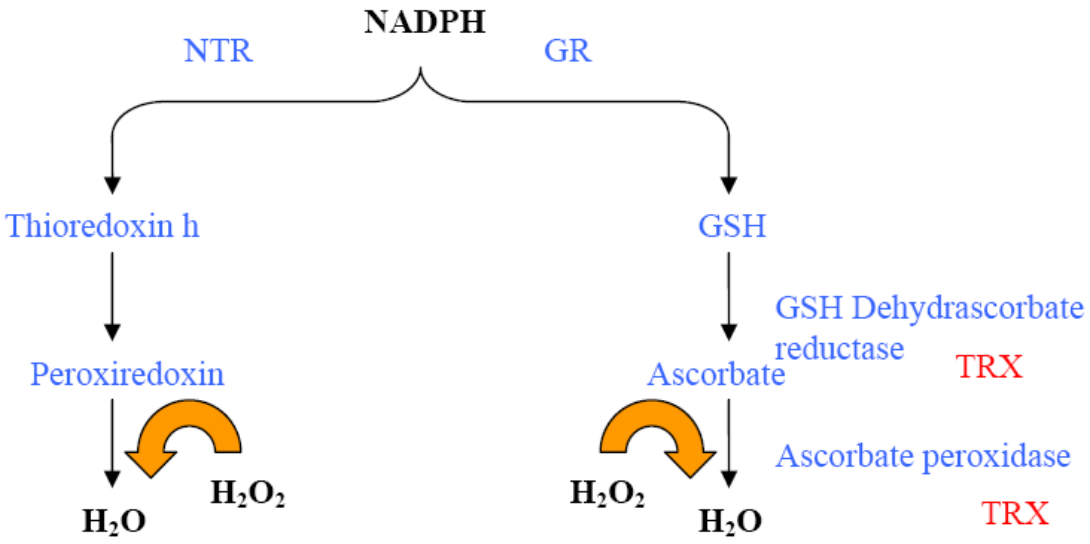


Figure 1.11: The role of thioredoxin h in oxidative stress response in the wheat endosperm. TRX in the diagram appears alongside the enzymes which are known thioredoxin targets. Diagram adapted from Wong *et al*., 2003 c.

Critical sulfhydryl groups or target proteins participating in the processes described in Figures 1.9 to 1.11 appear to become reversibly oxidized during grain maturation and

drying, this will result in either: the suppression of enzyme activity, the enhancement of inhibitor activity or the lowering of enzymatic accessibility. As a result of changing redox status of the endosperm, enzyme activity is reduced dramatically and the grain enters a quiescent state to preserve nutrients and energy for germination and subsequent growth. When the conditions are favourable and the seed begins to germinate thioredoxin is reduced and thus capable of reducing critical disulfide bonds of target proteins (Kobrehel *et al*., 1991).

A supporting observation for the role of redox in the regulation of germination is that a range of thiol-containing compounds such as thiourea, hydrogen sulphide, propyl mercaptan and 2,3-dimercaptopropanol have the ability to overcome profound dormancy in barley kernels, allowing ripe embryos to release cysteine and glutathione, which regulates many biochemical processes (Pheifer *et al*., 1995).

The components of a ferredoxin/thioredoxin system (ferredoxin, ferredoxin-thioredoxin reductase and thioredoxin) commonly described for chloroplasts has been found in amyloplasts isolated from the starchy endosperm of wheat (Balmer *et al*., 2006). Ferredoxin is reduced not by light as in chloroplasts but by metabolically generated NADPH (via ferredoxin-NADP reductase). Light is initially recognised as a thiol signal in the chloroplast, then as a sugar during transportation to the sink, where it can again be converted into a thiol signal thus allowing amyloplast reactions to be coordinated with photosynthesis in the leaves.

1.6.3. Phosphorylation of starch metabolic enzymes

Protein phosphorylation is key to the regulation of many proteins, in many cases this control involves interactions with scaffold 14-3-3 proteins (also known as protein 'adaptors') (Comparot *et al*., 2003). In plants 14-3-3 proteins have been isolated from many species including barley (Brandt *et al.*, 1992). 14-3-3 proteins are ubiquitous regulatory proteins which have a broad spectrum of activities and sub cellular locations, a common feature is the ability to interact with other proteins as binding partners (Palmgren *et al*., 1998; Fu *et al*., 2000). 14-3-3 proteins exist as saddle shaped homo- or heterodimers in which a broad central groove is able to bind to target proteins (Wu *et al*., 1997). Protein targets for 14-3-3 binding display a phosphorylated motif which is recognised by 14-3-3 proteins and forms part of the interacting domain. The most

characterised binding motifs are referred to as the mode 1 (R/KXXpSXP) and mode 2 (R/KXXXpSP) (pS = phosphorylated serine, X = any amino acid) (Muslin *et al*., 1996; Yaffe *et al*., 1997). Binding is dependent on localisation of 14-3-3 and target protein within the cell, phosphorylation status of the target protein and the steric availability of binding sites, binding may also occur to unphosphorylated ligands (Alam *et al*., 1994; Petosa *et al.*, 1998; Masters *et al*., 1999). Binding to 14-3-3 proteins can render targets active or inactive, binding to 14-3-3 dimers with multiple binding sites may be possible and result in bringing different proteins together, this has however not yet been observed in plants. 14-3-3 proteins have also been associated with changes in protein stability during periods of sugar starvation (Cotelle *et al*., 2000).

Nitrogen assimilation is an important step in plant metabolism, it involves the reduction of nitrate to nitrite by nitrogen reductase (NR) (Crawford, 1995). Control of NR expression is exerted by light and sucrose, expression is also finely regulated by the phosphorylation of the nitrate reductase inhibitor protein (NIP) (Bachmann *et al*., 1995). Purification by Bachmann *et al*. in 1996 demonstrated that NIP is composed of one or more 14-3-3 isoforms. Sucrose phosphate synthase catalyses the conversion of UDP-glucose and fructose-6-phosphate to sucrose-6-phosphate, the second last step in sucrose biosynthesis (Huber *et al*., 1996). 14-3-3 binding to sucrose phosphate synthase can result in either the activation (Moorhead *et al*., 1999) or inhibition (Toroser, 1998) of the enzyme.

Proteomic analysis of 14-3-3 proteins from developing barley grains revealed over 50 binding proteins, the majority of which were involved in carbohydrate metabolism and barley defence mechanisms (Alexander and Morris, 2006). Interestingly barley 14-3-3 binding proteins include granule bound starch synthase I, soluble starch synthase I, soluble starch synthase II, starch branching enzyme IIa, α-amylase, α-amylase inhibitor, β-amylase, sucrose synthase I, sucrose synthase II, fructose 1-6-bisphosphatase, sucrose phosphate synthase, sucrose phosphatase and alcohol dehydrogenase. In 2007 Stahl, *et al* noticed that the limit dextrinase inhibitor (LDI) protein contains a 14-3-3 binding motif. LDI binds to barley 14-3-3A and 14-3-3C in a phosphorylation dependent manner, binding of LDI to 14-3-3 proteins did not affect the limit dextrinase or LDI activities (Stahl *et al*., 2007).

1.6.4. Formation of multiprotein complexes

It is becoming apparent that the regulation of many cellular processes is achieved by the use of protein interaction domains, whereby polypeptides are associated to one another and in some cases to subcellular structures, small molecules or nucleic acids.

Examples include the amylase extender (ae) maize mutants which lack SBEIIb but also show a loss of SBEI and altered isoamylase type debranching enzyme activity (Colleoni *et al*., 2003). Mutations in maize which affect both pullulanase type debranching enzyme (*zpu1*-204) and an isoamylase type debranching enzyme (*su1-st)* both cause a loss in SBEIIa activity despite unchanged levels of the inactive polypeptide suggesting post-translational modifications and altered interactions with the debranching enzymes (James *et al*., 1995; Dinges *et al*., 2001, 2003). *Zpu1*-204 mutant also had decreased levels of β-amylase activity (Colleoni *et al*., 2003). Rice *ae* mutants lack SBEIIb, this phenotype caused a dramatic decrease in the activity of soluble SSI (Nishi *et al*., 2001). Barley *sex6* mutants have a loss of SSIIa activity in the developing endosperm, this results in the inability of SSI, SBEIIa and SBEIIb to bind within the granule matrix suggesting the formation of starch granule association protein complexes (Morell *et al*., 2003).

It has been suggested that to accomplish starch synthesis, debranching and synthase enzymes form a physical association within the amyloplast (Ball *et al*., 2003). Complexes formed in the wheat amyloplast have been shown to be dependent on key enzymes' phosphorylation status (Tetlow *et al*., 2004). Phosphorylation of SBEI, SBEIIb and Pho1 by plastidial protein kinase(s) resulted in the formation of a complex reversible by dephosphorylation (Figure 1.12). Physical interactions improve efficiency of processes as the product of one reaction can easily become the substrate of another while localized within the complex (substrate channelling).

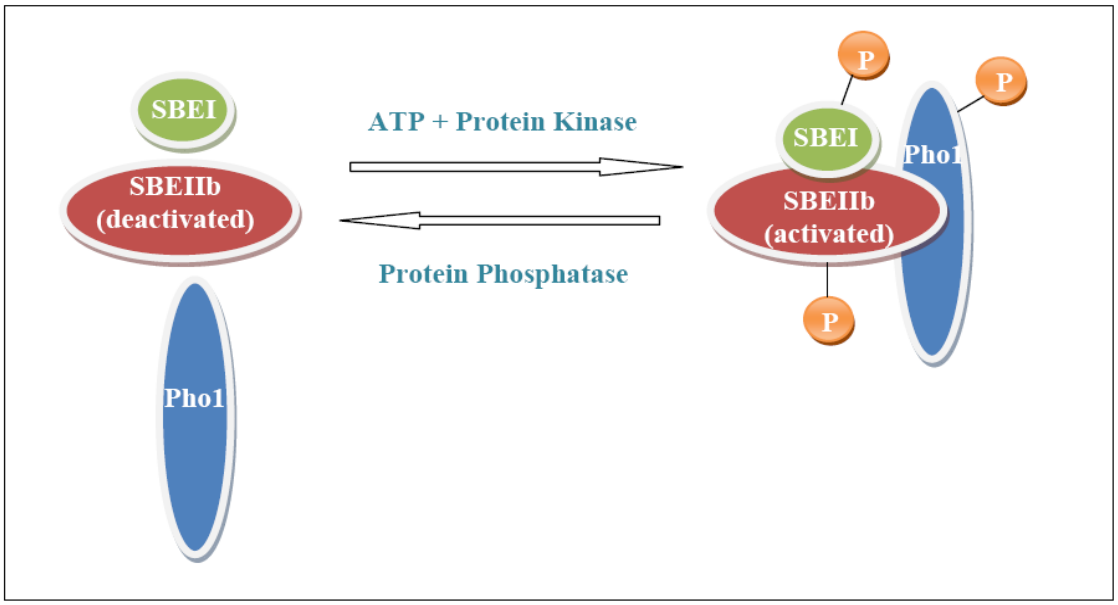


Figure 1.12: Proposed complex formation between SBEI, SBEIIb and Pho1 during starch biosynthesis. Diagram adapted from Tetlow *et al*., 2004.

1.7. Barley cell response to anaerobic stress

Anoxia and hypoxia are extremely stressful conditions for higher plants. All plant cell organelles including endoplasmic reticulum, polysomes and cytoplasm must undergo marked rearrangements in response to a lack of oxygen. Mitochondria are primary oxygen consumers, they suffer from oxygen deficiency prior to other organelles. In maize cells Ca^{2+} ions are released rapidly from the mitochondria into the cytosol following the onset of anoxia (Subbaiah *et al*., 1998), destructive, degradative rearrangements take place in mitochondrial membranes (Vartapetian *et al*., 2003). Aerobic mitochondrial cells are round or oval, cristae containing, dense matrices on transfer to anaerobic environment they arrest oxidative phosphorylation immediately, swell, decrease the number of cristae and the matrix gradually clarifies, following prolonged anoxia irreversible degradation of mitochondria and other organelles occurs eventually leading to lysis and cell death. Transfer of aerobically germinated rice seedlings to anoxia results in the appearance of mitochondria with stacks of parallel cristae and a dense matrix (Morisset, 1978).

1.7.1. Naturally occurring anaerobic stress on barley cells

Acidification of the barley starchy endosperm associated with maturation is correlated with the accumulation of malate and lesser amounts of citrate and lactate produced and secreted from the pericarp/testa/aleurone. Acidification is accompanied by rises in alcohol dehydrogenase (ADH) activity (Macnicol *et al*., 1992). ADH synthesis occurs in

mature aleurone tissue in low oxygen concentration, data suggests that conditions in the developing grain are consistent with hypoxia. In anoxic conditions the release of H^+ ions accompanying inorganic phosphate liberating hydrolysis of nucleoside triphosphate (NTP) causes the pH of the cytoplasm to decrease (Gout *et al*., 2001). H^+ extrusion is not sufficient to overcome this process. When cells are anoxic for short time periods, pH recovery is observed with increased concentrations of glucose, this can be attributed to the H^+ consuming ATP synthesis accompanying ethanolic fermentation (Vartapetian *et al*., 1976). Anaerobic mitochondria are responsive to external hexose and in germinating seedlings are dependent on the seeds' supply. Experiments on rice seedlings have shown that specific proteins are produced alongside alcohol dehydrogenase in response to anoxia (Maslova *et al*., 1976). Proteins expressed in anaerobic conditions appear to be associated with glycolysis, fermentation or the mobilization of carbohydrate reserves such as hexokinases, α-amylase and sucrose synthase (in rice and maize seedlings) (Sachs, 1993; Xia *et al*., 1995; Sachs *et al*., 1996; Perata *et al*., 1997; Rawyler *et al*., 1999; Saglio *et al*., 1999). During anoxic conditions the reduction of nitrate to nitrite and ammonia has been considered as a mechanism to compensate the oxidation of NADH and NADPH (Reggiani *et al*., 1985, 1993; Fan *et al.*, 1988; Oberson *et al*., 1999). In barley roots, anaerobic ethanol fermentation was not affected by increased nitrate reductase activity (Botrel *et al*., 1997).

1.7.2. Exploiting cell response to anaerobic stress in order to increase hydrolytic enzyme activity

Germinating barley under aerobic, followed by anaerobic conditions produced a malt with limit dextrinase activity which was 100 % free (i. e. no bound limit dextrinase) (M^{c}Cafferty *et al*., 2000). To achieve the maximum proportion of free limit dextrinase activity the grains were malted for 5 d aerobically and 3 d anaerobically. A malt mash was created whereby 20 % consisted of partially anaerobic malt with high free limit dextrinase proportions, this was an insufficient concentration to alter the limit dextrinase activity of the mash, inhibitors from the normal malt were found to inhibit the free limit dextrinase activity of the partially anaerobic malt.

1.8. Aims

The main indicators of malt quality include the recovery of a high fermentable sugar yield in the extract, the presence of yeast nutrients needed for satisfactory fermentation and that the malt delivers the flavour and process requirements of the brewer and

subsequent customers. The rate of barley grain endosperm modification depends on the distribution of moisture throughout the starchy endosperm, the rate of hydrolytic enzyme synthesis, the extent of release of hydrolytic enzymes into the endosperm and the structural features of the starchy endosperm which determine its resistance to digestion (Bamforth *et al*., 1983).

The main aims of this investigation were:

- To investigate the homogeneity of *Optic* malt nitrogen content, hydrolytic enzyme (α-amylase, β-amylase and limit dextrinase) activity and sugar content (glucose, fructose, sucrose, maltose and maltotriose) of 5 d aerobically germinated grains.
- To compare the nitrogen content, hydrolytic enzyme activity and sugar content of 5 d aerobic malt produced via micromalting at Heriot-Watt University brewing lab to 5 d aerobic malt produced commercially at Simpsons Maltings, Berwick-Upon-Tweed.
- To investigate the effect of 1 d anaerobiosis following 5 d aerobic micromalting on nitrogen content, hydrolytic enzyme activity and sugar content.
- To observe enzyme expression patterns of α-amylase, β-amylase and limit dextrinase during *Optic* barley grain development and germination.
- To purify limit dextrinase inhibitor protein in order to observe the effect the thioredoxin system has on the limit dextrinase: limit dextrinase inhibitor complex.

Chapter 2. Materials and methods

2.1. Materials

Chemicals, reagents and enzymes whose origin is otherwise unspecified in the methods were obtained from Sigma (Dorset, UK), Fisher Scientific (Loughborough, UK) or BDH (Poole, UK) and were of molecular biological grade or equivalent. All chemicals, enzymes and other materials were handled and stored according to manufacturers recommendations. The names of suppliers, chemicals, enzymes and kits used in this work are given in Table 2.1 and the suppliers of kits used in this work are listed in Table 2.2, homepages of suppliers are listed in Appendix B.

Suppliers	Chemicals, reagents and enzymes
GE Healthcare Buckinghamshire, UK	DNaseI, nitrocellulose membrane for western blotting
BioRad Hemel Hempstead, UK	acrylamide/bisacrylamide (29:1), TEMED, ammonium persulfate, molecular markers for SDS PAGE
Greiner Stonehouse, UK	1.5 ml, 15 ml and 50 ml polypropylene tubes Petri dishes
Helena Biosciences Sunderland, UK	Agarose
Kodak Sigma, Dorset, UK	X-ray film, developer and fixer solutions
MBI Fermentas Hanover, MD, USA	Restriction endonucleases, T4 DNA ligase, ribonuclease inhibitor, calf intestinal alkaline phosphatase, λ/*Hin*dIII DNA ladder
Oxoid Basingstoke, UK	agar, tryptone, yeast extract
Promega Madison, USA	M-MLV reverse transcriptase
Roche Lewes, UK	anti-digoxigenin antibody conjugated to alkaline phosphatase, blocking reagent, hexanucleotides, positively charged nylon membrane, anti-rabbit IgG conjugated to alkaline phosphatise
Whatman Maidstone UK	3 mm paper, filter paper No 1

Table 2.1: Suppliers of chemicals, reagents and enzymes used

Suppliers	Kits
BioRad	Protein assay kit
Millipore	DNA gel extraction kit
Megazyme	Total starch assay kit, β-glucosidase assay kit, α-amylase assay kit, β-amylase assay kit, limit dextrinase assay tablets
Roche	High pure PCR purification kit
Sigma	Thioredoxin reductase assay kit

Table 2.2: Suppliers of kits used

2.2. Centrifugation

Centrifugation of small volume samples were carried out in a Micro Centaur Microfuge (MSE) at maximum speed 11600 x g at room temperature unless otherwise cited. Larger sample volumes were centrifuged in a Sorval RC24 (DuPont) or BR401 (Denley) at stated speed and temperature.

2.3. Treatment and assessment of barley samples

2.3.1. Barley samples

Developing grains of variety *Golden promise* were harvested at various times post anthesis (as the anthers were becoming visible on the developing ear) during summer 2006 from Gogar Bank Farm, Edinburgh.

Mature barley and 5 d commercial malt of variety *Optic* were obtained from Simpsons Malt, PLC. Berwick-Upon-Tweed. Using a standard set of barley sieves, samples were cleaned from dust, chaff, broken grains and other seeds. Grains which were smaller than 2.2 mm in size were removed. Tests were then carried out on the barley to ensure it was within brewing quality parameters (see sections 2.3.2 to 2.3.7).

2.3.2. Weight of barley samples

40 g barley were weighed in triplicate and the number of grains in each lot was counted by hand, the 1000 grain weight was then worked out by simple proportion (Institute of Brewing recommended methods of analysis, 1997).

100 single grains, for each sample analysed, were weighed individually and these weights used to take an average grain weight which could then be used to modify subsequent assays for single grain analysis.

2.3.3. Germination of barley samples and water relations

Samples were assessed in triplicate for their germination capabilities (Essery *et al*., 1955). Germinative energy was measured, Petri dishes were lined with filter paper, 100 grains were placed on the paper and treated with 4 ml H_2O, 8 ml H_2O, 4 ml 1 mM GA_3 or 8 ml 1 mM GA_3. Petri dish lids were replaced to prevent moisture loss and the dishes were incubated at 20 ^oC. Chitted grains were counted after 24, 48 and 72 h. Cumulative counts from dishes treated with 4 ml H_2O are referred to as germinative energy and counts made from grains treated with 8 ml H_2O are referred to as water sensitivity.

The germinative capacity was also calculated using the hydrogen peroxide method (MacLeod, 1950). 200 grains were steeped for 72 h in 200 ml of 7.5 g l^{-1} hydrogen peroxide at 18 ^oC. The hydrogen peroxide was then removed, replaced with fresh solution and incubated for a further 24 h. Unchitted grains which had not developed both root and acrospire growth were separated and counted. The germinative capacity was calculated as described in Figure 2.1.

$$\text{Germinative capacity } (H_2O_2) = \frac{(200-n)}{2}$$

n = Number of grains without both root and acrospire growth

Figure 2.1: Equation used to calculate the germinative capacity of barley

2.3.4. Micromalting of barley samples

Barley samples were steeped in a steeping vessel of a Seeger Micromalting unit (Seeger Machinenfabrik, Fellbach, Germany). Steeping was carried out at 16 °C, the steeping regime was 8 h steep, 16 h air rest and 24 h steep. Samples were then germinated in the same micromalting unit for 6 d at 16 °C. Samples were mixed twice a day to avoid root matting and heterogeneity of modification. 500 g samples were removed every day, 300 g of which were kilned at 65 °C for 16 h. The remaining 200 g green malt was frozen at - 70 °C. The sample removed directly following steeping is referred to as 0 d germination.

For anaerobic grain an additional 500 g grain was malted for 5 d, removed and tightly packed into plastic containers which when full were made as air tight as possible using 'Nescofilm' to seal lids. Containers were incubated at 37 °C as was specified by M^{c}Cafferty *et al*., 2000 and Dixon's enzymic malt (patent 582,423, November, 1946). Samples were removed at various time periods and either kilned or frozen at – 70 °C.

2.3.5. Moisture content of malted barley samples

Malt samples were finely ground (smaller than 0.2 mm) using a Buhler-Miag mill. 5 g of malt flour was transferred to a pre-weighed dish. The flour-filled dish was dried at 105 ± 0.5 °C for 3 h and re-weighed. Moisture was calculated as described in Figure 2.2.

$$\text{Moisture (\%)}\ \frac{m}{m} = \frac{(W1-W2)}{W1} \times 100$$

W1 = Sample weight prior to drying (g)

W2 = Sample weight following drying (g)

Figure 2.2: Equation used to calculate the moisture content (%m/m) of samples during malting

2.3.6. Friability and homogeneity of malted barley samples

Friability is defined as the amount of energy required to mill malt. Grains with a greater degree of modification will have greater degradation of endosperm cell walls making the grain softer and thus easier to mill.

Friability and homogeneity were calculated as specified by section 2.17 of the Institute of Brewing recommended methods of analysis (1997). 50 g malt (in triplicate) was milled through/over the mesh screen of the friabilimeter (Pfeuffer) for 8 minutes at constant pressure. Particles from well modified malt passed through the screen while under-modified particles remained in the drum above the screen. The sample retained in the drum of the friability meter (non-friable fraction) was retained and weighed (Palmer, 1990). Friability was calculated as described in Figure 2.3.

$$\text{Friability (100 \%)} = 100 - (2 \times P)$$

$$P = \text{Mass of non-friable fraction (g)}$$

Figure 2.3: Equation used to calculate friability (%) of barley malt samples

Grains which were over ¾ whole (sustaining less than 25 % damaged, referred to as glassy grains) were manually removed from the non-friable fraction and weighed. The % glassy grains was calculated as described in Figure 2.4.

$$\text{Glassy grains (\%)} = 2 \times B$$

$$B = \text{Mass of grains which were less than 25 \% damaged}$$

Figure 2.4: Equation used to determine the % of glassy grains in the non-friable fraction of barley malt samples

Glassy grains were returned to the non-friable fraction. The non-friable fraction was then sieved through 2.2 mm slotted sieve. The particles which do not pass through the

sieve are referred to as partly unmodified grains. The % partly unmodified grains was calculated as shown in Figure 2.5.

Partly unmodified grains (PUG %) = 2 x C

C = mass of fraction retained on 2.2 mm sieve (g)

Figure 2.5: Equation used to calculate the % partially unmodified grain in non-friable fraction

Homogeneity of grain modification can then be calculated as shown in Figure 2.6.

Homogeneity (%) = 100 – PUG

PUG = partially unmodified grain (%) as calculated in figure 2.5

Figure 2.6: Equation used to calculate the % homogeneity of grain modification

2.3.7. Mashing of malted barley samples

Mashing methods were obtained from the Institute of Brewing recommended method of analysis (1997) section 2.3 – hot water extracts of ale, lager and distilling malts and were adjusted to facilitate smaller volumes. Malt was ground in a Buhler-Miag mill (setting 7). 10 g malt flour was added to a stainless steel mashing beaker and mashed with 75 ml pre-heated distilled H_2O at 65 ^oC for 1 h in a CM4 mashing bath (Canongate Technology LTD, Edinburgh). Clock glasses were placed on the top of the beakers to prevent moisture loss and slurry was continuously stirred using a magnetic stirring rod. The mash was then cooled for 30 m until about 20 ^oC, mash volume was adjusted to 103 ml with distilled H_2O. Resulting wort was filtered through an Ederol 12 (32 cm diameter) fluted filter paper. The first 10 ml filtrate was returned to the filter paper.

2.3.8. Hot water extract of mashed barley liquor

Specific gravity of the wort was determined by injecting 10 ml wort sample into a calculating digital Density meter (Stanton Redcroft PAAR DMA 46) and calculated using the equation shown in Figure 2.7.

Hot water extract (HWE $l^{o}kg^{-1}$) = 1000 x (SG – 1) x 10.13

SG = Specific gravity of filtrate at 20 ^{o}C.

Figure 2.7: Equation used to determine the hot water extract of the mash liquor ($l^{o}kg^{-1}$)

2.4. Analysis of malt and wort carbohydrate composition

2.4.1. Total starch content of malted barley

Total starch of malt samples was analysed using the amyloglucosidase/α-amylase assay procedure described by Megazyme (Co. Wicklow, Ireland). The total starch analysis procedure proceeds in two phases, the first phase involves the partial hydrolysis and total solubilisation of starch. The second phase utilizes amyloglucosidase activity to hydrolyse dextrins to glucose (M^{c}Cleary *et al*., 1994). Malt extracts contained glucose and maltosaccharides and were thus pre-treated to remove these compounds.

0.1 g malt flour was added to 5 ml 80 % (v/v) aqueous ethanol and incubated at 80 to 85 ^{o}C for 5 m. The slurry was vortexed and a further 5 ml 80 % (v/v) ethanol was added, the mixture was then centrifuged at 1000 x g for 10 m and the supernatant discarded. The pellet was resuspended in 10 ml 80 % (v/v) ethanol and vortexed, the centrifugation step was repeated. 3 ml thermostable α-amylase (300 U) stock solution (1 ml α-amylase (3000 U) diluted in 30 ml MOPS buffer (50 mM MOPS pH 7.0, 5 mM $CaCl_2$ and 0.02 % (w/v) sodium azide) was added to the pellet, which was then vigorously vortexed and incubated in a boiling water bath for 6 m. The mixture was allowed to cool to 50 ^{o}C in a water bath, 4 ml 200 mM sodium acetate buffer pH 4.5 was added followed by 0.1 ml amyloglucosidase (20 U), the tube was vortexed and incubated at 50 ^{o}C for 30 m. The volume of the solution was adjusted to 10 ml, tubes

were centrifuged at 1000 x g for 10 m, 1 ml supernatant was diluted in 10 ml distilled H_2O. 0.1 ml of the diluted supernatant was added to 3 ml GOPOD reagent (glucose determination reagent: 12 000 U L^{-1} glucose oxidase, 650 U L^{-1} peroxidase, 0.4 mM 4-aminoantipyrine in 20 ml 1 M potassium phosphate buffer with 0.22 M p-hydroxybenzoic acid and 0.4 % (w/w) sodium azide, pH 7.4) and incubated at 50 ^{o}C for 20 m. The absorbance was then measured at 510 nm. Glucose controls consist of 0.1 ml glucose standard solution (1 mg ml^{-1} on 0.2 % (v/v) benzoic acid) and 3 ml GOPOD reagent. Reagent blank consisted of 0.1 ml water and 3 ml GOPOD reagent all samples were read against this reaction blank. The total starch (% w/w) was calculated as described in Figure 2.8.

Total starch content = Abs_{510} x (F/W) x 90

Abs_{510} = Absorbance of reaction read against GOPOD reaction blank.

F = Glucose control = 100 (µg glucose)/Abs_{510} (of 100 µg glucose)

W = Weight of flour sample analysed

Starch (% w/w) = Total starch x 100/100 – moisture content (%)

Figure 2.8: Equation used to calculate starch content (% w/w) of malted barley samples

2.4.2. Fermentable sugars of wort

1 ml wort was diluted in 10 ml 0.1 M MES buffer pH 5.5. 1 ml diluted wort was analysed for amount of glucose, sucrose, fructose and maltose by high performance liquid chromatography (HPLC). HPLC was performed by Heriot-Watt technical support staff Jim Mackinlay. HPLC is a separation technique used to quantify various compounds in a solution; it is based on solutes partitioning between a liquid and solid phase. Carbohydrates were partially ionised at high pH and subsequently separated by anion exchange. Separation was achieved by high performance anion exchange (HPAE) and carbohydrates were detected using a pulsed amperometric detector (PAD) which detects the electrical current generated by carbohydrate oxidation on the surface of a gold electrode. Instruments used included a Dionex PAD (pulsed electrochemical detector) with gold electrode, a Gilson 302 pump, a Gilson 305 pump, a Gilson 802 Manomeric module, a Gilson 811B dynamic mixer and a Hewlett Packard degas module, data was analysed with the Hewlett Packard Chemstation data handling

(HP3365) package. Columns used were the Dionex Carbopac PA-1 Guard column, 4 x 50 mm and the Dionex PA-100 column, 4 x 250. Calibration standards consisted of sucrose, glucose, fructose (BDH >99 % pure) and maltose (Fluka) at 25 mg l^{-1} and maltotriose (Sigma) at 6.25 mg l^{-1}. Retention time for sugars analysed are shown in Table 2.3 and the eluent gradient programme on Table 2.4.

Retention time (min)	**Component**
5.66	Glucose
6.59	Fructose
9.20	Sucrose
16.37	Maltose
21.99	Maltotriose

*(Retention time may fluctuate slightly depending on room temperature).

Table 2.3: Retention time of wort sugar components on a Dionex PA-100 column

10 µl of internal standard solution (600 mg l^{-1} cellobiose) was added to 240 µl of calibration standard or sample to give final concentration of 24 mg l^{-1}.

Time (min)	% A (HPLC grade water)	% B (500 mM NaOH)
0	97	3
10	78	22
25	40	60
25.1	20	80
30	97	3

Table 2.4: Table showing eluent gradient programme for sugars on a Dionex PA-100 column

2.5. Analysis of nitrogen and hydrolytic enzyme activity of malt and wort

2.5.1. Total soluble nitrogen content of wort

Total soluble nitrogen of wort includes all amino acids, ammonia, peptides and proteins. 1 ml wort was diluted with 100 ml 5 g l^{-1} NaCl solution (Haslemore and Gill, 1995). The absorbance of the diluted wort was measured at both 215 and 225 nm against a NaCl blank using a quartz cuvette and a UV visible spectrophotometer with deuterium light (Ultraspec II, Biochron) and the total nitrogen calculated as shown in Figure 2.9.

$$\text{Wort soluble nitrogen (mg } l^{-1}) = 2249 \times (Abs_{215} - Abs_{225}) + 30$$

Figure 2.9: Equation used to calculate the total soluble nitrogen content (mg l^{-1}) of wort

2.5.2. Free amino nitrogen content of wort

This assay gives an estimate of amino acids, ammonia and the terminal α-amino nitrogen groups of peptides and proteins (Institute of Brewing recommended methods of analysis, 1997). 1 ml wort samples were diluted to 100 ml H_2O (~ 1 to 3 mg amino

nitrogen l^{-1}). 2 ml diluted wort was added to 1 ml colour reagent (0.55 M $Na_2HPO_4.12H_2O$, 0.44 M KH_2PO_4, 3 g fructose, 1 l water, 0.5 g ninhydrin/100 ml fresh, pH 6.6) and boiled for 16 minutes. Following boiling, the solution was allowed to cool for 20 minutes, once cool 5 ml of dilution reagent was added (9 mM KIO_3, 600 ml water, 400 ml 96 % ethanol) and the absorbance read at 570 nm. The standard was prepared by diluting 107.2 g glycine in 100 ml water and stored at 0 °C. Free amino nitrogen level of wort was calculated as shown in Figure 2.10.

Free amino nitrogen (FAN) (mg l^{-1}) = Abs^{s}_{570} x 2 x dilution/ Abs^{o}_{570}

Abs^{s}_{570} = absorbance of substrate at 570 nm, Abs^{o}_{570} = absorbance of standard at 570 nm

Figure 2.10: Equation used to calculate free amino nitrogen content (mg l^{-1}) of wort

2.5.3. β-glucanase activity of malt

β-glucanase activity of malt samples was ascertained using the Azo-barley glucan method (Megazyme). Malt extract is incubated with Azo-barley glucan substrate under defined conditions. The dyed substrate is depolymerised by malt β-glucanase to fragments which precipitate in solution. On centrifugation of the precipitant-treated reaction mixture the absorbance at 590 nm of the supernatant can be directly related to the level of malt β-glucanase (M^{c}Cleary *et al*., 1987).

8 ml extractant buffer (40 mM sodium acetate, 40 mM sodium phosphate, pH 4.6) was added to 0.5 g malt flour and vortexed prior to a 15 m extraction at room temperature. The slurry was then centrifuged at 1000 x g for 10 m. 0.5 ml supernatant (pre-incubated at 30 °C) was added to 0.5 ml aliquots of pre-warmed Azo-barley glucan substrate solution (1 % (w/v) dye-labelled beta-glucan in 0.02 % (w/v) sodium azide) and incubated at 30 °C for 10 m. Following incubation 3 ml precipitant solution (40 g sodium acetate, 4 g zinc acetate in 150 ml distilled H_2O, pH adjusted to 5 with HCl and the volume to 200 ml. 800 ml methyl cellosolve (methoxyethanol) was added and vortexed. Mixtures were allowed to stand for 5 m prior to centrifugation 1000 x g for 10 m. The absorbance of the supernatants was measured at 590 nm against a reaction blank. β-glucanase activity was calculated as shown in Figure 2.11.

β-glucanase activity (U kg^{-1}) = U (per 0.5 ml extract) x 16 x 2000

U per 0.5 ml extract were obtained from a standard curve relating absorbance to β-glucanase activity (U kg^{-1}) = 630 x Abs + 4

16 as from a total extract of 8 ml 0.5 ml were used in this assay

2000 is a weight correlation factor. 0.5 g of malt are extracted, results are expressed as units per kg malt.

Figure 2.11: Equation used to calculate the β-glucanase activity (U kg^{-1}) of malt flour

2.5.4. α-amylase activity of malt

The level of α-amylase in barley grains is a key quality parameter; elevated levels in mature barley are used as an indication of pre-harvest sprouting, α-amylase also plays an important role in starch hydrolysis. α-amylase activity was measured using amylazyme red tablets (Megazyme). The substrate employed is Azurine cross-linked amylose which is prepared by dyeing and cross-linking high amylose starch to produce a material which hydrates in water but is water insoluble. Hydrolysis by α-amylase produces red-dyed fragments, the rate of solubilisation of which (increase in absorbance at 510 nm) can be directly related to enzyme activity (M^{c}Cleary *et al*., 1989 b).

1 g malt flour is suspended in 20 ml Buffer A (100 mM sodium maleate, pH 6.0, 5 mM calcium chloride and 0.02 % (w/v) sodium azide), mixed by inversion and allowed to extract for 15 m at room temperature. The slurry was centrifuged at 1000 x g for 10 m. A 0.5 ml aliquot of the supernatant was diluted in 50 ml buffer A. An amylazyme red tablet was added to pre-heated (40 ^{o}C, 5 m) diluted malt extracts and incubated at 40 ^{o}C for 10 m. Following incubation 10 ml 2 % (w/v) Trizma base was added to terminate the reaction and solutions vortexed. The samples were left for 5 m at room temperature, revortexed and filtered through Whatman No 1 (9 cm) filter paper. The absorbance at 590 nm was measured against a reaction blank (an amylazyme red tablet was added after the Trizma base). α-amylase activity was calculated as shown in Figure 2.12.

α-amylase activity (U g^{-1}) = mU/ assay x (1/100) x 100 x dilution

mU/assay was determined by reference to the appropriate standard curve relating absorbance at 590 nm to α-amylase activity.

1/1000 = conversion from mU to U

100 = initial extraction volume (100g per g solid)

Dilution = further dilution of the initial extraction solution

Figure 2.12: Equation used to calculate α-amylase activity (U g^{-1}) of malt

2.5.5. Total β-amylase activity of malt

β-amylase activity was determined using the Betamyl method (Megazyme). The manufacturers' instructions were adhered to but, adjusted for the measurement of single grains. β-amylase plays a central role in the complete conversion of starch to fermentable sugars during the malting of barley grains. The assay is based on the ability of β-amylase and inability of α-amylase to rapidly hydrolyse the substrates p-nitrophenyl-α-D-maltopentaose (PNPG5) and p-nitrophenyl-α-maltohexaose (PNPG6). The betamyl reagent consists of PNPG5 and high purity α-glucosidase, on hydrolysis of PNPG5 to maltose and p-nitrophenyl maltotrioside by β-amylase, the p-nitrophenyl maltotrioside is immediately cleaved to glucose and free p-nitrophenol by the α-glucosidase present in the substrate mixture. The rate of release of p-nitrophenol relates directly to the rate of release of maltose by β-amylase. The reaction is stopped and the phenolate colour developed on addition of Trizma base solution (M^{c}Cleary *et al*., 1989 a).

5 ml extraction buffer (50 mM Trizma base, 1 mM di-sodium EDTA, pH 8) was added to 0.5 g malt flour, the slurry was vortexed and extracted for 1 h at room temperature (with frequent vortexing throughout extraction period). The slurry was then centrifuged at 100 x g for 10 m. 0.2 ml extract was added to 10 ml dilution buffer (100 mM maleic acid, 1 mM di-sodium EDTA, 1 mg ml^{-1} BSA and 0.02 % (w/v) sodium azide), 0.2 ml of this solution was further diluted in 5 ml dilution buffer.

0.2 ml of suitably diluted, pre-equilibrated (40 ^{o}C, 5 m) supernatant was added to 0.2 ml pre-equilibrated betamyl substrate solution (47.5 mg *p*-nitrophenyl maltopentaoside PNPG5 and 1000 U α-glucosidase in 10 ml), samples were then incubated at 40 ^{o}C for 10 m. 3 ml stopping reagent (1 % (w/v) Trizma base) was then added to samples and vortexed. The absorbance of samples at 410 nm were read against a reaction blank (Trizma added prior to betamyl reagent). β-amylase activity was calculated as shown in Figure 2.13.

$$\beta\text{-amylase activity (U g}^{-1}) = (Abs_{410}/10) \times (3.4/0.2) \times (1/17.8) \times (10/1) \times 1250$$

$$= Abs_{410} \times 1194$$

Abs_{410} = absorbance of reaction at 410 nm – absorbance of reaction blank at 410 nm

10 = incubation time (m)

3.4 = total volume in cell (ml)

0.2 = aliquot assayed (ml)

17.8 = E_{mM} ρ-nitrophenol in 1 % Trizma base

10 = extraction volume (ml g^{-1})

1250 = dilution (fold)

Figure 2.13: Equation used to calculate β-amylase activity (U $^{g-1}$) in malt

2.5.6. Limit dextrinase activity of malt

Limit dextrinase activity was measured using limit dextrizyme tablets (Megazyme). The substrate is azurine linked pullulan which is hydrolysed by limit dextrinase and pullulanase but, resistant to degradation by other commonly occurring amylolytic enzymes. The tablet is a dyed, cross linked polysaccharide which rapidly hydrates in water but, is water insoluble. Hydrolysis of the dyed pullulan produces water soluble dye fragments, the rate of increase of which (increase in absorbance at 590 nm) can be related directly to enzyme activity (M^{c}Cleary, 1992).

0.25 g malt samples were suspended in 4 ml extraction buffer (100 mM sodium maleate, pH 5.5, 0.2 % (w/v) sodium azide with or without 25 mM DTT) and extracted at 40 °C for 5 h. Slurries were then centrifuged at 1000 x g for 10 m. A limit-dextrizyme tablet was added to 0.5 ml pre-equilibrated (40 °C, 5 m) supernatant and allowed to incubate for 10 m at 40 °C. After this time 5 ml 1 % (w/v) Trizma base was added to terminate the reaction and the samples vortexed. Samples were allowed to stand for 5 m at room temperature prior to vortexing filtering them through Whatman No 1 (9 cm) filter paper. The absorbance of the filtrate at 590 nm was measured against a reaction blank (Trizma base added prior to addition of limit-dextrizyme tablet). Limit dextrinase activity was calculated as shown in Figure 2.14.

Limit dextrinase activity (mU kg^{-1}) = mU/assay x (1/1000) x 32000

mU/assay is determined by reference to the standard curve relating absorbance at 590 to limit dextrinase activity (mU kg^{-1})

1/1000 = conversion from mU to U

32000 = conversion from mU/0.5 ml extract to per kg malt flour

Figure 2.14: Equation used to calculate limit dextrinase activity (mU kg^{-1}) of malt

2.6. Single malt grain analysis

It has been well documented that standard methods of malt analysis give very little indication of the homogeneity of individual endosperm modification. The distribution of ß-glucan breakdown and ß-glucanase development in single grains have been extensively studied (de Sa and Palmer, 2004; Home *et al*., 1997; Home *et al*., 1999; Palmer, 1999; Palmer, 2000). The activity of hydrolytic enzymes in single malted barley grains is unknown, hydrolytic activity is crucial to endosperm modification particularly in terms of starch breakdown and the carbohydrate composition of wort prior to fermentation. Existing methods of analysis have been adapted in this study to analyse the homogeneity of starch breakdown and the hydrostatic power of the malt.

2.6.1. Single malt grain mashing

100 single grains were individually ground using a pestle and mortar. Flour from single grains (average grain weight 0.037 g) was placed in 1.5 ml conical screw cap microtubes and 0.36 ml Millipore water was added, the slurry was vortexed and incubated for 1 h at 65 ^{o}C. Following incubation the volume was adjusted to 0.5 ml with the addition of 0.15 ml Millipore water, allowed to cool and centrifuged for 5 minutes. The supernatant (mash) was stored at – 20 ^{o}C.

2.6.2. Fermentable sugar content of single malt grains

0.5 ml mash was added to 0.75 ml MES buffer (0.1 M MES at pH 5.5). The total amount fermentable sugars in single grains was analysed by adding 1.25 ml mash dilution to 0.067 ml Millipore water (n = 20). All fractions were placed into a rapidly boiling water bath for 7 minutes, cooled, recentrifuged and analysed by HPLC for glucose, fructose, sucrose, maltose and maltotriose content.

2.6.3. Total soluble nitrogen (TSN) content of single malt grains

TSN of 100 single grains was determined by diluting 0.2 ml wort in 85 mM NaCl (Haselmore *et al*., 1995). The absorbance of the solution was read at 215 and 225 nm and total soluble nitrogen calculated as previously described in Figure 2.11.

2.6.4. α-Amino nitrogen content of single malt grains

0.1 ml wort was diluted to 10 ml Millipore water. 2 ml diluted wort was then used as described in section 2.5.2 and Figure 2.12 to calculate the α-amino nitrogen content of single grains.

2.6.5. α-amylase activity of single malt grains

The amylazyme red assay was carried out as previously described, manufacturer's instructions were followed but adapted for single grain (average weight = 0.037 g) analysis. 100 single grains were ground in a pestle and mortar and placed in 1.5 ml microfuge tubes, 0.74 ml extraction buffer was added and α-amylase extracted and assayed for as described in section 2.5.4. α-amylase activity of single grains was calculated as shown in Figure 2.15.

α-amylase activity (mU grain^{-1}) = mU/assay x 1.48

1.48 = conversion factor from 0.5 ml assay volume to 0.74 ml extraction volume.

Figure 2.15: Equation used to calculate α-amylase activity of single malt grains (mU grain^{-1})

2.6.6 β-Amylase activity of single malt grains

100 single grains were ground in a pestle and mortar. 0.037 g fine powder obtained was extracted 0.37 ml extraction buffer and assayed as described in section 2.5.5. β-amylase activity of single grains was calculated as shown in Figure 2.16.

β-amylase activity (U grain^{-1}) = U/assay x 1.85

1.85 = conversion factor from 0.5 ml assay volume to 0.616 ml original extract volume

Figure 2.16: Equation used to calculate β-amylase activity of single malt grains (U grain^{-1})

2.6.6. Limit dextrinase activity

The Megazyme instructions for the limit-dextrizyme method were adhered to with alterations making the assay suitable for single grain analysis. 200 5 d aerobically germinated single grains were ground in a pestle and mortar to a fine powder (average grain weight = 0.037 g) and extracted for 5 h in 0.616 ml extraction/activation buffer. 100 grains were used to determine 'free' activity and 100 grains extracted in the presence of DTT were used to determine 'total' activity. Following extraction samples were centrifuged at for 10 mins, 0.5 ml aliquots of supernatant were removed and assayed as previously described, and the activity (mU/assay) was worked out using a standard curve based on ceralpha control malt flour (Megazyme) activity against Abs_{590}. The limit dextrinase activity of single grains was calculated as shown in Figure 2.17.

Limit dextrinase activity (mU grain^{-1}) = mU/assay x 1.232

1.232 = conversion factor from 0.5 ml assay volume to 0.616 ml original extract volume

Figure 2.17: Equation used to calculate the limit dextrinase activity of single malt grains (mU grain^{-1})

2.7. Recombinant RNA and DNA methods

2.7.1. Isolation of total RNA from plant tissue

RNA was isolated using an adaptation of the lithium chloride method (Cathala *et al*., 1983). 0.5 g barley grain was frozen in liquid nitrogen and ground in a pre-chilled pestle and mortar. The flour was suspended in 500 µl autoclaved REB buffer (25 mM Tris-HCl, 25 mM EDTA, 75 mM NaCl, 1 % (w/v) SDS, pH 8.0) and vortexed. 500 µl water-saturated phenol was added to the suspension which was then vortexed and centrifuged at 13 000 rpm for 5 m. The upper aqueous layer was added to 500 µl autoclaved water-saturated phenol, vortexed and centrifuged. The upper aqueous layer was added to 500 µl chloroform, vortexed and centrifuged. 0.25 volumes of autoclaved DEPC-treated 10 M lithium chloride was added to the upper aqueous layer slowly while mixing. The resulting solution was then incubated overnight at – 20 ^{o}C and centrifuged at 13 000 rpm for 15 m. The supernatant was discarded and the pellet dried and resuspended in 100 µl autoclaved dH_2O.

2.7.2. Ethanol precipitation of RNA

10 µl 3 M autoclaved DEPC-treated Na Acetate (pH 5.5) and 250 µl 96 % (v/v) ethanol were added to 100 µl RNA solution. It was then vortexed, incubated on ice for 10 m and centrifuged at 13 000 rpm for 10 m. The resulting pellet was resuspended in 50 µl autoclaved dH_2O.

2.7.3. Quantifying amount of RNA

The amount of RNA present was determined by diluting 4 µl RNA in 400 µl dH_2O and measuring the absorbance in a quartz cuvette at OD_{260} and OD_{280} against a dH_2O blank on a spectrophotometer (Ultrospec II, LKB Biochrom). RNA amounts were

calculated as shown in Figure 2.18. Contamination of RNA solutions by protein is measured at an absorbance of 280 nm, the ratio of OD_{260}/OD_{280} should fall between 1.8 and 2.0 (depending on pH) to be of an acceptable level. Levels lower than this indicate that re-extraction is required to remove proteins.

$$\text{Amount of RNA } (\mu g\ ml^{-1}) = OD_{260} \times 2 \times 100 \times 40$$

Figure 2.18: Equation used to calculate the amount of RNA present in a sample

2.7.4. Agarose gel electrophoresis of RNA

All surfaces, pipettes and plates were pre-treated with RNaseZAP (Sigma). 1 % (w/v) agarose gels were prepared in 1 x MOPS buffer (10 x MOPS buffer contained 200 mM MOPS, 50 mM NaAc, 10 mM EDTA, pH 7.0 and was autoclaved), microwaving until all agarose was dissolved, cooling and adding 10 ml 37 % (v/v) formaldehyde per 100 ml gel solution. RNA was added to 2 x volume RNA loading buffer (50 % (v/v) formamide, 16 % (v/v) formaldehyde, 10 % (v/v) 10 x MOPS buffer, 0.1 mg ml^{-1} ethidium bromide and 0.01 % (w/v) bromophenol blue), heated to 70 ^{o}C for 10 m, cooled on ice. Following the casting and setting of the gel 5 or 10 µg RNA samples in loading buffer were loaded into wells and run in 1 x MOPS running buffer at 100 v for 30 m. Resulting bands were visualised via a UV transilluminator and photographed with a UVP gel documentation system.

2.7.5. Preparing cDNA from total RNA

Autoclaved DEPC-treated dH_2O was used to bring 2 µg RNA up to a final volume of 5 µl. 10 µl DNase stock solution (3 µl 0.1 M DTT, 1 µl 10 x DNase I buffer, 0.5 µl porcine ribonuclease inhibitor at 30 U μl^{-1}, 5.2 µl autoclaved DEPC-treated dH_2O and 0.3 µl DNase I at 10 U μl^{-1}) was added, mixed and incubated at 37 ^{o}C for 1 h to destroy genomic DNA contamination. To denature the DNase the mixture was then incubated at 70 ^{o}C for 10 m and cooled on ice. The first cDNA strand was then synthesised by adding 14 µl of RT mix (6 µl 5 x RT buffer, 3 µl 0.1 M DTT, 0.3 µl 10 x hexanucleotides, 0.6 µl 25 mM dNTPs, 1 µl porcine ribonuclease inhibitor at 30 U μl^{-1}, 3.3 µl DEPC-treated dH_2O) incubating at 37 ^{o}C for 5 m, adding 0.8 µl M-MLV reverse transcriptase at 200 U μl^{-1}, incubating for a further 5 m at room temperature and then incubating for 1

h at 42 ^{o}C. Following incubation the reverse transcriptase was inactivated by heating at 75 ^{o}C for 10 m and subsequently cooling on ice.

2.7.6. Isolation of barley genomic DNA

Purification of barley genomic DNA was carried out using a modification of the Dellaporta *et al*. (1983) method. One barley embryo was ground in a pestle and mortar in 750 µl extraction buffer (50 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 1 % (w/v) SDS, 10 mM 2-mercaptoethanol, pH 8.0) and incubated at 65 ^{o}C for 10 m. 5 M potassium acetate was added and the suspension incubated on ice for 20 m. The extract was then centrifuged at 13 000 rpm for 10 m, an equal volume of isopropanol was added to the supernatant and the precipitate centrifuged at 13 000 rpm for 2 m. The resulting supernatant was discarded, the pellet was washed in 500 µl 70 % (v/v) ethanol and recentrifuged at 13 000 rpm for 10 m. The pellet was dissolved in 20 µl dH_2O.

2.7.7. Agarose gel electrophoresis of DNA

DNA was separated by electrophoresis using the Mupid-2 Mini Gel Migration Trough system (Cosmo Bio Co., Ltd). 1 % (w/v) agarose was dissolved in 0.5 x TBE (10 x TBE contains 890 mM Tris-HCl, 890 mM Boric acid, 20 mM EDTA at pH 8.0) by heating in a microwave. On cooling to ~ 60 ^{o}C ethidium bromide was added (0.5 µg ml^{-1}) and the gel poured and allowed to set in a casting tray with comb provided with the system. DNA samples were mixed with 6 x loading buffer (60 % (v/v) glycerol, 60 mM EDTA, 0.09 % (w/v) bromophenol blue, 0.09 % (w/v) xylene cyanol) and loaded onto the gel with molecular weight marker (λ/*Hind*III) giving detectable fragments at 23130, 9416, 6557, 4361, 2322, 2027 and 564 bp. Electrophoresis was carried out in running buffer 0.5 x TBE at 100 v for 30 m.

2.7.8. Quantifying amount of DNA

The amount of DNA was determined by comparison with λ/HindIII molecular weight marker whereby the amount of DNA (ng) of bands were known.

2.7.9. Amplification of DNA by PCR

PCR primers were designed using the Primer3 web based program (Appendix B). Standard PCR reactions were carried out in a 0.2 ml thin walled PCR tube (Greiner) and contained 2 µl DNA (~ 10 ng plasmid DNA or ~ 100 ng genomic DNA or ~ 100 ng cDNA), 1 µl of each primer at 20 pmol μl^{-1} (forward and reverse), 8 µl 1.25 mM dNTP, 5 µl 10 x PCR buffer (0.67 M Tris-HCl, 0.16 M $(NH_4)_2SO_4$, 0.1 % (v/v) Tween 20) and 1 µl of *Taq* polymerase (1 U) brought up to a final volume of 50 µl with d H_2O. Tubes were placed in a thermal cycler (Gene Amp® PCR system 2700, Applied Biosystems). The PCR reaction cycle consisted of 5 min at 94 ^oC prior to the addition of *Taq* polymerase, followed by 30 cycles of denaturation at 94 ^oC for 30 s, annealing for 30 s at primer dependent temperature, extension of 1 m at 72 ^oC, an additional extension time of 7 m at 72 ^oC and cooling at 4 ^oC.

2.7.10. PCR product purification

PCR products were purified using the QIAquick PCR purification kit (Qiagen). 5 x volumes of PB buffer were added to PCR sample and mixed. The resulting mixture was placed in a QIAquick spin column with a 2 ml collection tube and centrifuged at 3000 rpm for 1 m. The flow-through was discarded and 0.75 ml PE buffer was then added to the column and centrifuged at 13 000 rpm for 1 m. The flow through was again discarded and the spin column centrifuged at 13 000 rpm for 1 m. The DNA was then eluted from the column by adding 50 µl EB buffer (10 mM Tris-HCl, pH 8.5) and centrifuging at 13 000 rpm for 1 m.

2.7.11. Construction of 'T-vectors'

1 µg pBluescript SK plasmid (Stratagene) (Figure 2.19) was digested with 10 U blunt-end restriction enzyme Eco321, 2 µl 10 x restriction buffer and 0.1 mg ml^{-1} in a total volume of 20 µl for 60 to 120 m at 37 ^oC. The digest was then cleaned by mixing with 1 x volume of 50:50 TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffered phenol/chloroform, the mixture was vortexed and centrifuged at 13 000 rpm for 10 m. The upper aqueous layer containing the DNA was removed and mixed with an equal volume of chloroform to remove any residual phenol, this mixture was then vortexed and centrifuged at 13 000 rpm for 10 m. The upper aqueous layer containing nucleic acids was precipitated from solution by the addition of 0.1 x volume 3 M Na Acetate at pH 5.2 and 2 x volumes 96 % (v/v) ethanol. The solution was mixed and incubated for 30 m at -20 ^oC and centrifuged at 13 000 rpm for 10 m. The pellet was then washed

with 1 x volume 70 % (v/v) ethanol and recentrifuged for 10 m. The supernatant was discarded and the pellet dried and re-dissolved in 1 x *Taq* PCR buffer (Promega) to give 1 µg DNA in 20 µl. 2 mM dNTP and 1 U *Taq* polymerase was added and the mixture incubated at 72 ^{o}C for 2 h. Following incubation the mixture was extracted once in phenol: chloroform and once in chloroform and precipitated in ethanol as previously described. The resulting pellet containing nucleic acids was dried and dissolved in TE buffer at an approximate concentration of 25 ng μl^{-1}. (Marchuk *et al*.,1990).

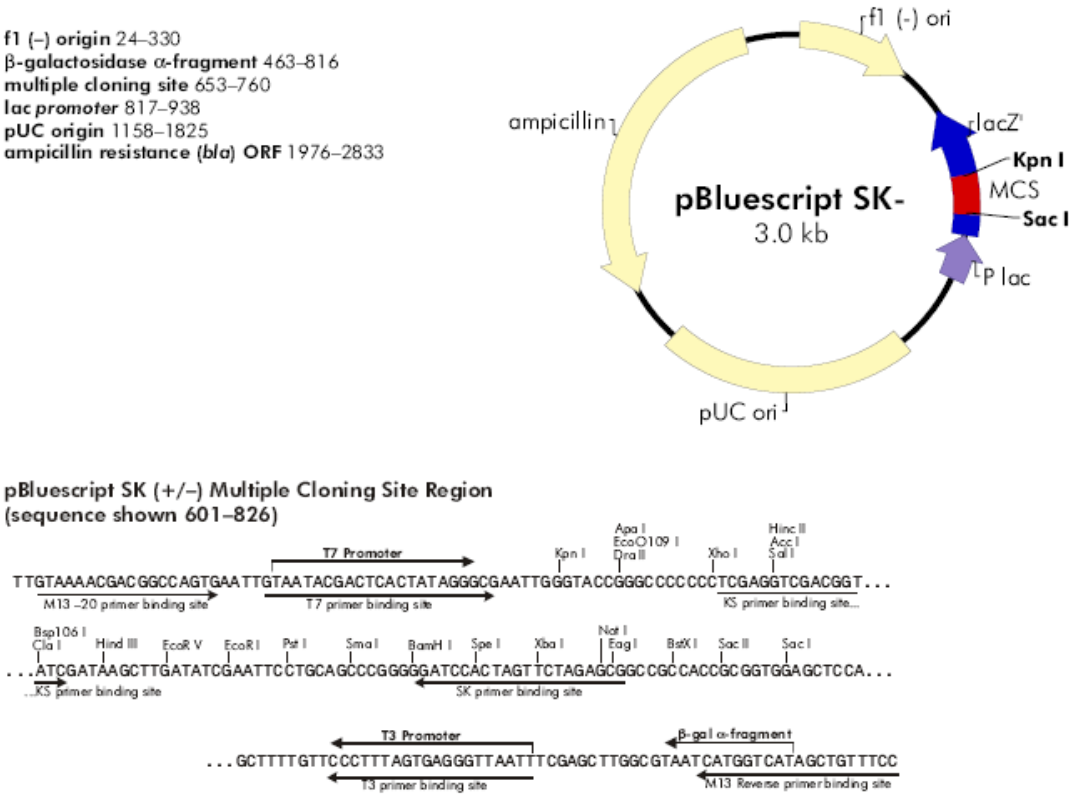


Figure 2.19: Map of pBluescript SK plasmid used and sequence of multiple cloning site region, Stratagene

2.7.12. Ligation of DNA fragments

For ligations 25 ng T-vector and sufficient PCR product to give a 3:1 insert: vector molar ratio was used. 2 µl 10 x ligation buffer (0.4 M Tris-HCl, 0.1 M $MgCl_2$, 0.1 M DTT, 5 mM ATP, pH 7.8) and 5 U T4-ligase (Fermentas) were added to the restriction digested vector and insert mixture in a total volume of 20 µl adjusted by dH_2O. The ligation mixture was incubated at 20 ^{o}C overnight. The ligase was then deactivated by heating at 75 ^{o}C for 15 m (Ausubel *et al*., 1989).

2.7.13. Preparation of competent *E.coli* cells

50 ml LB broth (1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl, pH 7.0, autoclaved) was inoculated with a single colony of *Escherichia coli* (DH5α of XL-1blue, Figure 2.20) and incubated overnight at 37 °C. 4 ml of this culture were then diluted in 400 ml LB broth and grown at 37 °C until an absorbance at OD_{590} of 0.375 was obtained. This culture was then aliquoted into pre-chilled 50 ml centrifuge tubes, incubated on ice for 10 m and then centrifuged at 1600 x g for 10 m at 4 °C. The pellets obtained were carefully resuspended in 10 ml ice cold $CaCl_2$ solution (60 mM $CaCl_2$, 15 % (v/v) glycerol, 10 mM PIPES, pH 7.0, autoclaved), incubated on ice for 30 m and centrifuged at 1100 x g for 5 m at 4 °C. Each cell pellet was resuspended in 2 ml ice cold $CaCl_2$ solution, 200 µl aliquots were then frozen in liquid nitrogen and stored at -70 °C.

***E.coli* strain**	**Genotype**
XL1-Blue MRF'	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173, *endA*1, *supE*44, *thi-1*, *recA*1, *gyrA*96, *relA*1, *lac*[F'*proAB lacI*^{q} *Z* ΔM15 Tn10 (Tet^R)]

Table 2.5: Strain of E.coli used and its genotype (Stratagene)

2.7.14. Transformation of competent *E.coli* cells

Competent *E.coli* cells were transformed by heat shock. A 200 µl aliquot of competent cells were thawed on ice. 5 µl ligation mixture was added to the cells, which were then incubated on ice for 20 m. The cells were then heat shocked at 42 °C for 2 m. 0.5 ml of LB broth was added to the cells and incubated at 37 °C for 1 h. The cell suspension was centrifuged at 2000 x g for 3 m, the supernatant was removed and the pellet resuspended in approximately 50 µl.

2.7.15. Blue white selection

Plates were prepared (1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl, 1 % (w/v) agar, pH 7.0, autoclaved) with 100 µg ml^{-1} ampicillin. 100 µl 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.238 g IPTG dissolved in 8 ml dH_2O, brought up to 10 ml with dH_2O, filter sterilised to 0.22 µm in 1 ml aliquots, stored at -20

^{o}C) and 20 µl 20 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (X-gal stock solution was prepared by dissolving 100 mg X-gal in 5 ml dimethyl formamide, stored at -20 ^{o}C) were spread onto the plates and allowed to dry. 100 µl transformed *E.coli* cell suspension was spread onto the X-gal containing plates, which were then incubated overnight at 37 ^{o}C. After incubation the plates were placed at 4 ^{o}C for 2 h. White colonies were indicative of recombinant plasmids and blue colonies showed non-recombinant plasmids.

2.7.16. Restriction enzyme digestions of DNA

1 to 2 µg plasmid DNA was digested with 10 U appropriate restriction enzyme, in 2 µl restriction buffer specific for restriction enzyme and 0.1 mg ml^{-1} RNase A in a total volume of 20 µl adjusted with dH_2O. The digest was carried out for 60 to 90 m at enzyme dependent temperature (generally 37 ^{o}C). Table 2.6 shows restriction enzymes used.

Restriction enzyme	**Buffer composition**	**C**
***Hin*dIII**	10 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg ml^{-1} BSA, pH 8.5	37
***Pvu*II**	10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 0.1 mg ml^{-1} BSA, pH 7.5	37
EcoRI	50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 0.2 % Triton x-100, 0.1 mg ml^{-1} BSA, pH 7.5	37
***Sal*I**	50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl0.1 mg ml^{-1} BSA, pH 7.5	37

Table 2.6: Restriction enzymes used, the required 10 x buffer composition and optimum temperatures

2.7.17. Preparation of DIG-labelled RNA probes

A standard PCR reaction using M13 F and M13 R was carried out on cloning vectors containing the desired DNA fragment for preparation of the probe. Either T7 or T3 RNA polymerase (Fermentas) was used to produce a single stranded RNA from the PCR product depending on the orientation of the cloned insert in respect to T7 or T3 phage promoter sites. For detection of a sense gene, the RNA probe must be in antisense direction. 2 µl 5x RNA buffer (0.4 M Tris-HCl, 60 mM $MgCl_2$, 0.1 M DTT, 0.1 M NaCl and 20 mM spermidine, pH 7.9), 0.5 µl porcine RNase inhibitor, 1 µl DIG RNA labelling mix (Roche) and 1 µl T7 or T3 RNA polymerase was added to 200 to 500 ng PCR product and the mixture made up to 10 µl with autoclaved dH_2O. The reaction was incubated at 37 ^oC for 2 h.

2.7.18. Northern blotting

Total RNA was separated on an RNA gel as previously described and a photograph of ethidium stained RNA taken under UV light. The gel was then rinsed in DEPC-treated 20 x SSC (3 M NaCl, 0.3 M Na citrate, pH 7.0) for 15 m to remove the formaldehyde. A blotting stack was assembled, all paper used in the blotting stack was pre-wet in autoclaved 20 x SSC. The blotting stack consisted of ~ 6 cm thick stack of paper towels, 2 pieces of Whatman 3 mm paper (slightly larger in size than the gel) were placed on top of the paper towels, a nylon membrane the same size as the gel was wet in water prior to soaking in DEPC-treated SSC and placed on top of the 3 mm paper. The loading wells were removed from the gel which was placed on top of the membrane. 2 pieces of 3 mm paper were placed on top of the gel and 2 long pieces of the 3 mm paper were used to create a bridge over the gel into a 20 x SSC containing buffer tank. The assembly was covered in cling film to prevent evaporation and the stack allowed to blot overnight at room temperature. Following blotting the RNA was fixed to the membrane using a UV crosslinker (UVC-508, Anachem) emitting 120 000 µJ short wavelength UV light. The blot was washed in dH_2O to remove excess salt and air dried.

2.7.19. Hybridisation and detection

The RNA blot was prehybridised for 1 h in hybridisation buffer (6 M urea, 6 x SSC, 1 % (w/v SDS) and 50 mM Tris pH 7.5) using a rotating roller bottle in a hybridisation oven (Hybaid). The DIG labelled RNA probe was denatured at 95 ^oC for 10 m. The blot was then incubated in hybridisation buffer containing 100 ng ml^{-1} denatured DIG RNA

probe at 68 ^{o}C overnight. The membrane was washed in twice in 2 x SSC containing 0.1 % (w/v) SDS at 65 ^{o}C for 30 m (low stringency wash) and then in 0.2 x SSC containing 0.1 % (w/v) SDS at 65 ^{o}C for 30 m (high stringency wash). The membrane was briefly washed in DIG1 buffer (100 mM Tris pH 8.0, 1 M NaCl, autoclaved) and incubated for 1 h in DIG2 buffer (blocking buffer: 0.1 M maleic acid, 1 M NaCl, 0.3 % (v/v) Tween, pH 8.0). The blot was then incubated for 30 m at room temperature with gentle shaking in DIG3 buffer (DIG2 buffer with 1: 10000 anti DIG conjugated to alkaline phosphatase, Roche). The blot was washed with 50 ml DIG1 buffer, 4 washes were carried out, each wash was for 10 m. The blot was incubated for 5 m in DIG4 buffer (detection buffer: 0.1 M Tris, 0.1 M NaCl, pH 9.5, autoclaved). 0.5 to 1 ml CPD-star substrate was added to the membrane, the membrane was then exposed to X-ray film for 30 s to 30 m (depending on the signal intensity).

2.7.20. X-ray film development

All steps were carried out in a dark room using a red safety light. Fixer and development solutions supplied by Kodak were diluted 1:5 in dH_2O as specified in the manufacturer's instructions. The exposed X-ray film was placed into developer solution and shaken gently until a signal could be seen, it was then rinsed in water and placed into fixer solution for 3 m. The film was rinsed in water and then allowed to dry.

2.8. Protein methods

2.8.1. Partially purified Limit dextrinase

Limit dextrinase was partially purified from barley using part of a method described by Kristensen *et al*., 1998. Optic barley was micromalted, the steeping regime included 8 h full steep, 16 h air rest and 24 h full steep at 16 ^{o}C, malting took place over 9 d at 16 ^{o}C. Malt was kilned over 3 d at 30 ^{o}C and ground to < 0.2 mm gap. 500 g flour was extracted at room temperature for 2 to 4 h in 4 l 0.2 M sodium acetate with 5 mM ascorbic acid buffer at pH 5.0 with slow stirring and left overnight at 4 ^{o}C. The slurry was then centrifuged for 10 minutes at 10 000 x g at 4 ^{o}C, the supernatant was saturated to 20 % with ammonium sulphate (106 g l^{-1}). Following re-centrifugation the supernatant was saturated to 70 % ammonium sulphate (312 g l^{-1}) and centrifuged for 15 minutes at 4 ^{o}C. The resulting pellet was dissolved in 0.5 M $CaCl_2$ and 50 mM sodium acetate at pH 5.0, the suspension formed was desalted by dialysis against two

changes of 20 mM sodium acetate at pH 5.0 overnight (membrane molecular weight cut off was 30 kDa, Serva). The dialysis membrane was prepared beforehand by boiling for 7 minutes in an excess of 10 mM sodium bicarbonate and incubation for several minutes in 10 mM Na_2 EDTA. The membrane was also washed several times in dH_2O and stored in 50 % (v/v) ethanol. The dialysed protein solution was then clarified by centrifugation 2000 x g for 15 minutes at 4 ^oC and the resulting supernatant vacuum filtered through 0.2 µm filter (Millipore). DEAE Sephacel (Pharmacia) ion exchange column connected to an FPLC system (fast performance liquid chromatography system, Pharmacia) was equilibrated with 20 mM sodium acetate at pH 5.0. The clarified protein solution was loaded onto the column and unbound proteins were washed off with 20 mM sodium acetate buffer pH 5.0. Elution of bound proteins was carried out with the same sodium acetate buffer with a linear salt gradient of 0 to 1 M NaCl at a flow rate of 120 ml/h over 1 h, 2 ml fractions were collected. Fractions with limit dextrinase activity (determined using red pullulan) were pooled and precipitated with 2 M ammonium sulphate.

2.8.2. Protein content of extracts and column fractions

The protein content of limit dextrinase and inhibitor samples from each stage of purification was determined using the Protein assay kit (Biorad) based on the Bradford dye binding procedure (Bradford, 1976) according to the manufacturer's instruction, using a standard curve based on BSA.

2.8.3. Limit dextrinase activity

Activity was determined using red pullulan as a substrate as described by Kristensen *et al*. (1998). Aliquots of limit dextrinase extracts were made up to a total volume of 200 µl with dilution buffer, 0.2 M sodium acetate, 5 mM $CaCl_2$ and 0.1 % (w/v) BSA at pH 5.0, and assayed with 100 µl 2 % (w/v) red pullulan (Megazyme) in 0.5 M KCl. The reaction took place at 40 ^oC over 20 minutes and stopped with the addition of 600 µl 80 % (v/v) ethanol and vigorous vortexing. The mixture was left at room temperature for 10 minutes and centrifuged at 10 000 x g for 8 minutes. The absorbance of the supernatant was measured at 510 nm. One unit of limit dextrinase activity is defined as the amount that gives $(A^S_{510\ nm} – A^O_{510\ nm})/min = 1$. $A^S_{510\ nm}$ is the absorbance of the sample at 510 nm and $A^O_{510\ nm}$ is the absorbance of the reaction blank, which contained buffers only.

2.8.4. Limit dextrinase inhibitor activity

To assay extracts and column fractions for limit dextrinase inhibitor activity aliquots of each inhibitor pool were added to 50 µl partially purified limit dextrinase (activity ~ 4.5 mU) at appropriate volumes to show ~ 50 % inhibition (i.e. limit dextrinase activity per 300 µl of assay, made up to total volume with dilution buffer, was decreased from 4.5 mU to 2.25 mU), since the relationship between inhibitor concentration and % inhibition was found to be linear at this concentration of inhibitor (MacGregor *et al*., 1994 c). One inhibitor unit is defined as the change in mU of limit dextrinase activity per µg protein added (∆mU/ µg protein) under assay conditions defined.

2.8.5. Partially purified limit dextrinase inhibitor

Limit dextrinase inhibitor was extracted from barley *c.v. Optic* using a method by MacGregor *et al*. (1994 c). Barley was ground in a Buhler Miag mill to pass through a 0.2 mm screen. 250 g ground barley was extracted for 16 h at 4 ^{o}C in 750 ml 0.02 M sodium acetate buffer pH 5.2 with 25 mM DTT to ensure all limit dextrinase inhibitor is unbound. After centrifugation 10 000 x g at 4 ^{o}C for 15 minutes, the supernatant was heated to 80 ^{o}C for 30 minutes. The extract was cooled, recentrifuged and filtered through a 0.2 µm cellulose membrane. A CM Sepharose weak cation column (Pharmacia) was equilibrated with 0.02 M sodium acetate pH 5.0. After application of heat treated extract 120 ml/h the column was washed with equilibration buffer to remove all unbound proteins. Bound proteins were eluted from the column at 120 ml h^{-1} at a linear salt gradient of 0 to 300 mM NaCl. 10 ml fractions were collected and assayed for inhibition as described previously. Fractions containing activity were pooled and desalted using a PD-10 column according to the manufacturer's instructions (Amersham biosciences). The PD-10 desalting column has a Sephadex G-25 medium matrix and a particle size range of 85 to 260 µm, the elution buffer was 50 mM Tris pH 8.8. The desalted fractions in 50 mM Tris buffer pH 8.8 were loaded onto a pre-equilibrated MonoQ column linked to an FPLC (Pharmacia). Bound proteins were eluted from the column at 120 ml h^{-1}, 2 ml fractions were collected and assayed for inhibitor activity. Fractions containing activity were pooled and saturated to 100 % with ammonium sulphate (76.7 g l^{-1}) on ice for 30 minutes, following centrifugation the pellet was resuspended in 500 µl 0.02 M sodium acetate buffer at pH 5.2 (the eluent). The sample was centrifuged at 13 000 rpm for 2 minutes and the supernatant loaded onto a pre-equilibrated Superose 12 HR 10/30 gel filtration column. The sample was eluted at flow rate 0.5 ml min^{-1} and 0.5 ml fractions collected. At all stages of the

purification process SDS-PAGE and Western blotting was carried out on pooled fractions to ensure the presence of the inhibitor.

2.8.6. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

The discontinuous buffer system of Laemmli (1970) was used. The resolving gel solution containing 10 to 12 % (w/v) polyacrylamide (29:1 acrylamide: bisacrylamide was used throughout), 375 mM Tris-HCl at pH 8.8, 0.1 % (w/v) SDS, 0.1 % (w/v) ammonium persulfate and 0.05 % TEMED was poured between glass plates assembled on a gel former (Mighty Small, Hoefer). The gel solution was overlaid with water saturated isobutanol to ensure a flat surface. Following 30 minutes polymerisation the isobutanol was poured off and the stacking gel solution, 4 % (w/v) polyacrylamide, 125 mM Tris-HCl pH 6.8, 0.1 % (w/v) SDS, 0.05 % (w/v) ammonium persulfate and 0.1 % TEMED, was poured on top of the resolving gel, a comb was inserted to form loading wells and the gel allowed to polymerise for 30 minutes. The gel and glass plates were mounted into the assembled electrophoresis equipment, the comb was removed and the buffer tanks filled with 1 x SDS running buffer (24 mM Tris-HCl pH8.3, 192 mM glycine and 3.5 mM SDS). Samples were prepared by mixing with 2 x sample loading buffer (0.15 M Tris-HCl, 1.2 % (w/v) SDS, 60% (w/v) glycerol, 15 % (v/v) 2-mercaptoethanol, 0.09 % (w/v) bromophenol blue) in a 1:1 ratio (protein: buffer) and heated at 80 ^{o}C for 10 minutes. Samples were loaded onto the gel, electrophoresis was carried out at 200 V for 45 minutes. A molecular weight protein marker standard (BioRad) was run on each gel. Following electrophoresis the gel was removed from between the two plated and incubated for 1 h at room temperature while shaking in Coomassie Brilliant Blue solution (10 % (v/v) acetic acid, 50 % (v/v) methanol, 0.1 % (w/v) Coomassie Brilliant Blue R250 BioRad). The gel was then destained in destaining solution (10 % (v/v) acetic acid, 50 % (v/v) methanol) overnight at room temperature. Proteins were visible as blue bands on a clear background.

2.8.7. Western blotting

A freshly run unstained SDS-PAGE gel, 2 Scotch Brite pads, 4 pieces of 3 mm filter paper (Whatman) and a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) were initially pre-equilibrated in 1 x western running buffer (25 mM Tris-HCl, 192 mM glycine at pH 8.3). The blotting stack was assembled by placing 2 sheets of filter paper onto a Scotch Brite pad, the gel was placed on top of the filter paper and the nitrocellulose membrane onto the gel. The other sheets of filter paper were used to

cover the membrane followed by the second Scotch Brite pad. The gel holder was closed and inserted into the blocking chamber (BioRad) filled with 1 x western running buffer. Blotting was carried out at 350 am for 45 minutes with ice cooling and magnetic bar stirring. Following blotting the membrane was blocked in western blocking buffer (3% (w/v) skimmed milk powder, 0.5% TWEEN 20 in 1 x PBS buffer: 4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl) for 1 h at room temperature. The membrane was then incubated for 30 minutes in a 1:2000 dilution of high limit dextrinase inhibitor antibody solution in western blocking buffer. The membrane was washed 5 times (5 minutes per wash) in 1 x PBS buffer and incubated for 30 minutes in a 1:20000 dilution of anti rabbit IgG coupled to alkaline phosphatase (Roche) in western blocking buffer. The membrane was then washed 4 times in 1 x PBS buffer, the detection of the signal was then carried out colorimetrically. After incubation in the detection buffer the membrane was placed into the colour solution (45 µl NBT and 35 µl BCIP in 10 ml detection buffer) and developed in the dark at 37 ^{o}C. NBT solution contained 75 mg ml $^{-1}$ NBT in 70 % (v/v) dimethylformamide. BCIP solution contained 50 mg ml $^{-1}$ BCIP in 100 % (v/v) dimethylformamide. Washing the membrane and air drying of the blot stopped the reaction.

Chapter 3. Results

In this investigation *Hordeum vulgare* variety *Optic* was micromalted for 5 d aerobically in Heriot-Watt micromaltings and individual grains analysed in order to establish the degree of variability which exists in single malt grains. It is important to remember that barley and the malt produced from it is derived from natural living material and is hence subject to variation, which can occur as a result of genetic and environmental conditions.

5 d aerobic malt produced commercially at Simpsons maltings, Berwick-Upon-Tweed, was also examined as industrial malting methods differ from those used when micromalting. Due to these differences micromalting cannot be used as a model for what happens industrially. Standard malt specifications are not always accurate indicators of how well malt will perform during brewing. The variety of barley affects quality in terms of the percentage nitrogen or protein present in the grain, the percentage of β-glucan in the grain following malting, the enzyme activity and the size and homogeneity of the grain (O'Rourke, 2002 a). Malts showing very similar results from conventional analysis can exhibit unpredictable levels in brewhouse performance; this has been partially attributed to uneven modification of the endosperm, which is masked in an analysis on a bulk sample of malt (de Sá and Palmer, 2004).

Many proteins expressed in cereal seedlings during anaerobic conditions appear to be associated with glycolysis, fermentation and the mobilisation of starch reserves (i.e. hexokinases, α-amylase and starch synthase) (see section 1.6.2). In 2000 M^{c}Cafferty *et al.* produced malt with high 'free' limit dextrinase activity by micromalting for 5 d aerobically and 3 d anaerobically (see section 1.6.3). Unfortunately when this 5 d aerobic 3 d anaerobic malt was produced it had an unattractive odour similar to silage and thus could not be used to brew with due to the inevitable introduction of unfavourable smell and taste to the final product. In this study a 5 d aerobic 1 d anaerobic malt was used as this unpleasant odour was not present, 6 d aerobic micromalt was also analysed as a control. The aim was to see if a similarly high level of limit dextrinase would be observed in the 5 d aerobic 1 d anaerobic malt and what, if any difference this would make on the fermentable sugar levels as well as the amylolytic enzyme activities. The study of anaerobic malt is also of commercial importance, as due to the potential lack of rotation of grains at the bottom of malting

beds (depending on bed depth) it is possible that a proportion of grains will be subjected to anaerobic conditions.

Enzyme assays are often based on average results of malt flour and can thus conceal heterogeneity or the different distribution values of individual grains. Even modification (the breakdown of endosperm cell structure making starch granules accessible) and the production of fermentable sugars by subsequent enzyme activity during the malting process are essential in the production of good quality malt. Single grain analysis is of commercial importance as it allows the maltster to predict how the malt will perform in the brewhouse taking into account not only an average but, the single grain distribution of the variable, for example the number of grains which have high and low values.

The initial goal in this investigation was to investigate the viability of the barley variety used and to establish that adequate modification had taken place during malting. In order to investigate heterogeneity at the single grain level, the following parameters were investigated: nitrogen levels, fermentable sugar levels, hydrolytic enzymes α-amylase, β-amylase and limit dextrinase activity. Expression patterns of α-amylase, β-amylase and limit dextrinase in bulk malt samples were also determined.

When analysing moisture content, friability, hot water extract and homogeneity, fermentable sugar levels, nitrogen levels and enzyme activities from a bulk sample only the aerobic micromalt was measured from 2 d to 5 d as the 6 d aerobic micromalt and the 5 d aerobic 1 d anaerobic micromalt were derived from this sample and it was assumed that the 5 d commercial ‘green’ malt met industrial specification during the period of malting.

Statistics used in this investigation to analyse data obtained from single grains are:

1. Mean: The most common measure of central tendency, the arithmetic average of a set of numbers.
2. Median: The value of the middle item when the data are arranged from lowest to highest, the middle value, the point whereby exactly half the data are above it and half below it.

3. Range: The length of the smallest interval which contains all the data, the difference between the highest and lowest data point.
4. Standard deviation: The square root of variance, measures the spread or dispersion around the mean of a data set.
5. % cv: The coefficient of variation, a measure of dispersion calculated by dividing the standard deviation of a distribution by its mean. The standard error of an estimate, expressed as a ratio or percentage of the estimate.
6. Cumulative distribution function (CDF): Describes the probability distribution of a real value random variable *X*. For every real number x, the CDF of X is given by:

$$x \rightarrow Fx(x) = P(X \geq x)$$

The right hand side represents the probability that the random variable *X* takes on a value less than or equal to x.

3.1. Assessment of *Optic* barley samples

Various parameters were measured to ensure that the *Optic* used in this study was viable and of commercial quality including 1000 grain weight (g), the germinative energy (% germination), the germinative capacity (% germination), moisture content of 'green malt' (% w/w) and the moisture content of kilned malt (% w/w). Measuring the β-glucanase activity (U kg^{-1}) and friability (%) of the *Optic* malt and calculating the homogeneity (%) from the friability (see section 2.3.6) gave indications to the degree of endosperm modification. Hot water extract (l^{o} kg^{-1}) provides an insight to the sugar content of the wort based on its specific gravity. Results are presented in sections 3.1.1 to 3.1.8.

3.1.1. Barley grain germination

Germination is defined as the ability of the barley grain to sprout. The germinative energy is the percentage of barley grains which can be expected to germinate under normal conditions. Water sensitivity is the germinative energy of a barley sample when the water in the germination environment is increased. The germinative capacity is the percentage of grains which can be expected to germinate under optimal conditions; H_2O_2 is used to provide a balance of water and oxygen. 1000 grain weight (g), germinative energy (% germination), water sensitivity (% germination) and germinative capacity of *Optic* barley used in this study are shown in Table 3.1. For malted barley industry specifications require that the minimum level of germinative energy and

capacity is 95 % (Palmer, 1989). It was found that the *Optic* sample used here were well within normal industrial specifications for germinative energy and capacity (Table 3.1).

1000 Grain weight (g)	39.7 ± 5.03
Germinative energy A (% germination)	96 ± 3
Germinative energy B (% germination)	99 ± 1
Water sensitivity A (% germination)	92 ± 6
Water sensitivity B (% germination)	92 ± 6
Germinative capacity (H_2O_2 test)	95 ± 3

Table 3.1: Assessment of *Optic* barley samples. Values are indicative of n = 3 ± standard deviation

Germinative energy A = % germination following 72 h in 4 ml H_2O

Germinative energy B = % germination following 72 h in 4 ml GA_3

Water sensitivity A = % germination following 72 h in 8 ml H_2O

Water sensitivity B = % germination following 72 h in 8 ml GA_3

3.1.2. Single grain weight

100 single grains of 5 d aerobic micromalt, 5 d aerobic commercial malt, 5 d aerobic 1 d anaerobic micromalt and 6 d aerobic micromalt were weighed and averages taken in order to adapt subsequent analysis protocols, downsizing them in order to make them suitable for smaller quantities. Single grain weights are shown in Figures 3.1 and 3.2, statistical analysis is shown in Table 3.2 and cumulative distribution curves are shown in Figure 3.3.

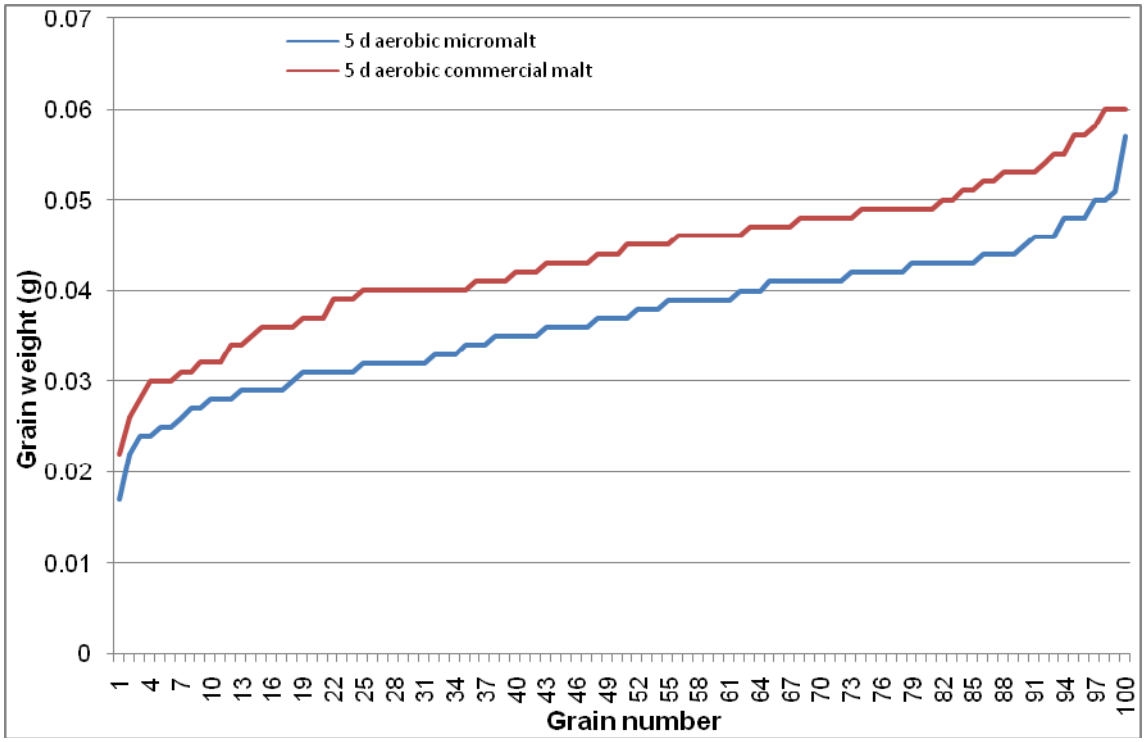


Figure 3.1: Grain weight (g) of 100 single kilned malt grains produced following 5 d aerobic germination in the micromalting unit at Heriot-Watt University and 5 d aerobic commercial malt produced at Simpsons Maltings, Berwick-Upon-Tweed

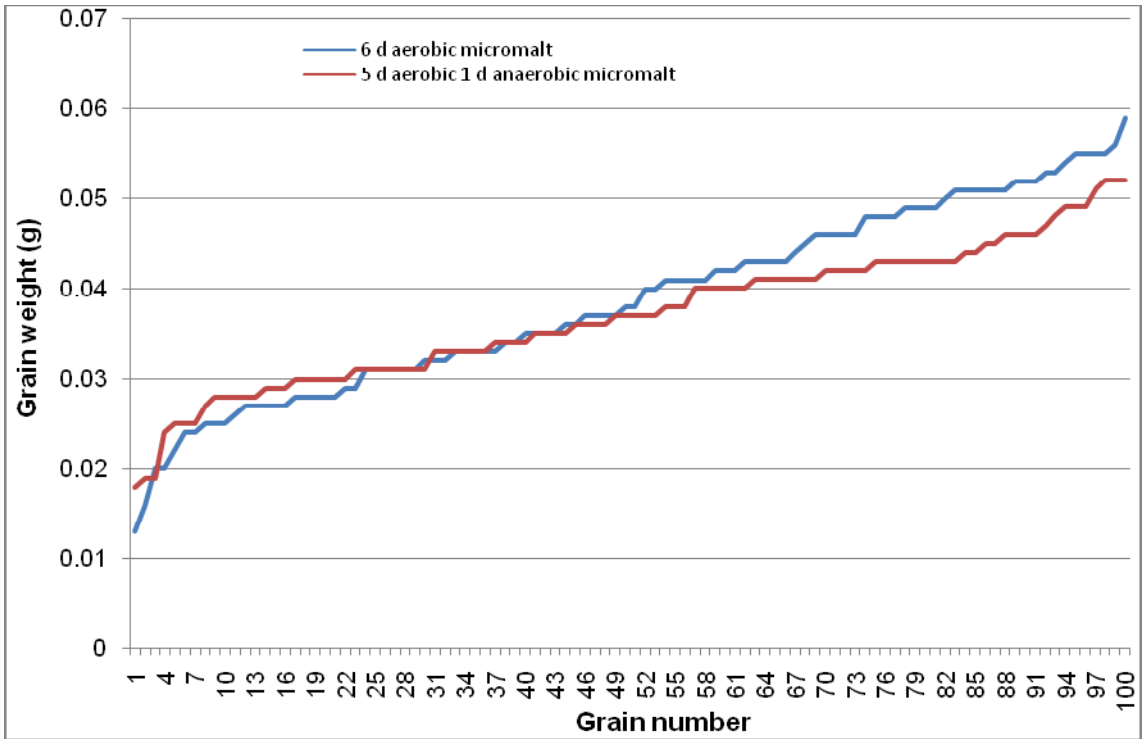


Figure 3.2: Grain weight (g) of 100 single kilned malt grains produced following 6 d aerobic micromalting and 5 d aerobic 1 d anaerobic micromalting

Statistic	5 d aerobic micromalt	5 d aerobic commercial malt	6 d aerobic micromalt	5 d aerobic 1 d anaerobic micromalt
Mean	0.037	0.044	0.039	0.037
Standard deviation	0.007	0.008	0.01	0.076
Median	0.037	0.044	0.038	0.037
Range	0.04	0.038	0.046	0.034
%cv	19.38	17.87	27	20.63

Table 3.2: Statistical analysis for single grain weight (g) of 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt

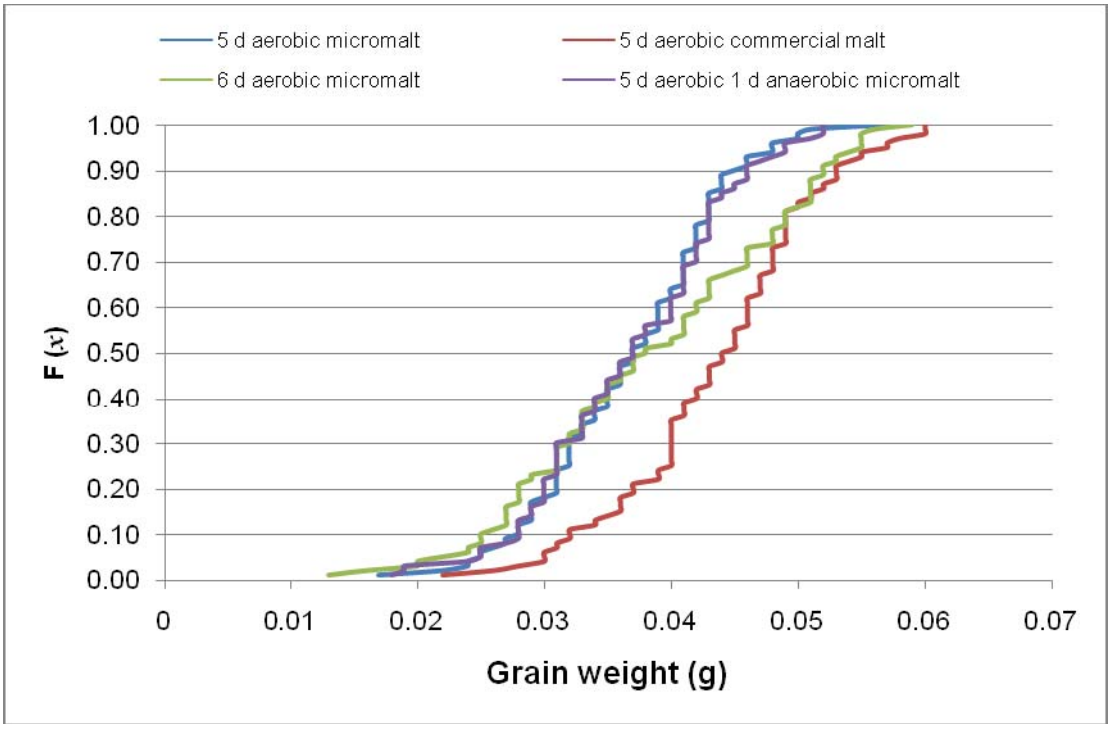


Figure 3.3: Cumulative distribution function (CDF) of single grain weight (g) for 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt

The 5 d aerobic commercial malt had the highest mean grain weight (Table 3.2), a large proportion of the grains weighed were heavier than values obtained for other malt samples. The 6 d aerobic micromalt had the second highest mean grain weight, with the highest range and standard deviation. The 5 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt had the same mean grain weight and median; the 5 d aerobic micromalt had a slightly higher range and slightly lower standard deviation than was observed for the 5 d aerobic 1 d anaerobic micromalt.

3.1.3. Moisture content of 'green malt' and kilned malt

Over the years the moisture content for brewers' and distillers' malt has gradually increased from under 4 % to as high as 5 % (w/w) (Bathgate, 1989). Kilning to very low moisture often results in malt with very low enzyme content and activity as prolonged high kilning temperatures often denature enzymes. The higher the moisture content of the malt the lower the energy consumption required to dry it. However malts with moisture content greater than 5 % (w/w) are difficult to mill and may result in undesirable 'green malt' odour notes being introduced into the beer or spirit. The moisture content of the 'green malt' was measured, the results show that by 5 d aerobic malting (120 h) the moisture content of the grain was ~ 46% (Figure 3.4) which is the expected level prior to kilning. The *Optic* malt was then kiln dried to ~4.5 % (w/w) as recommended by the Institute of Brewing for lager, ale and distillers malt (Table 3.3).

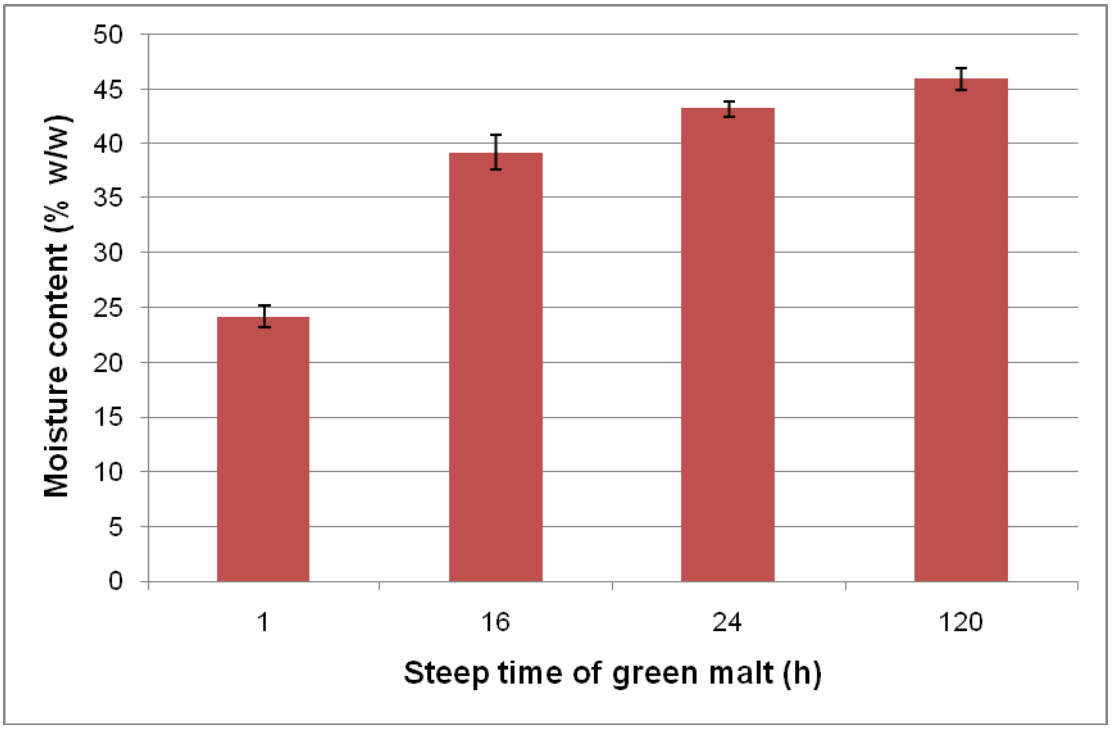


Figure 3.4: Moisture content (% w/w) of 'green malt' during the first 24 h steeping (steeping regime: 8 h steep, 16 h air rest, 24 h steep) and at 120 h from the initiation of steeping, values are indicative of n = 3 tests on same malt ± standard deviation

Malt Sample	Moisture content of kilned malt
5 d aerobic micromalted *Optic*	4.5
5 d commercially malted *Optic*	4.5
6 d aerobically micromalted *Optic*	4.5
5 d aerobic 1 d anaerobic micromalted *Optic*	4.5
IOB lager malt	4.5
IOB ale malt	4.0
IOB distillers' malt	4.0

Table 3.3: Moisture (% w/w), following kilning, of 5 d aerobic malt, 5 d commercial malt, 6 d aerobic malt, 5 d aerobic 1 d anaerobic malt as well as those expected by the Institute of Brewing for lager, ale and distillers' malt (IOB analyses taken from Palmer, 2006)

3.1.4. Friability and homogeneity

Friability and homogeneity of the malt are regarded as important factors in malt specification. Friability is the ability of the malt to be readily ground to a powder or flour and is an indicator of good modification; homogeneity is the uniformity of modification among grains in a sample. Malts are considered well-modified if friability is over 85 % and homogenous if homogeneity values are in the range 96 and 100% following 5 d malting (Palmer, 2006). Following 5 d aerobic micromalting (120 h) the *Optic* malt used in this study was sufficiently well modified (Figure 3.5).

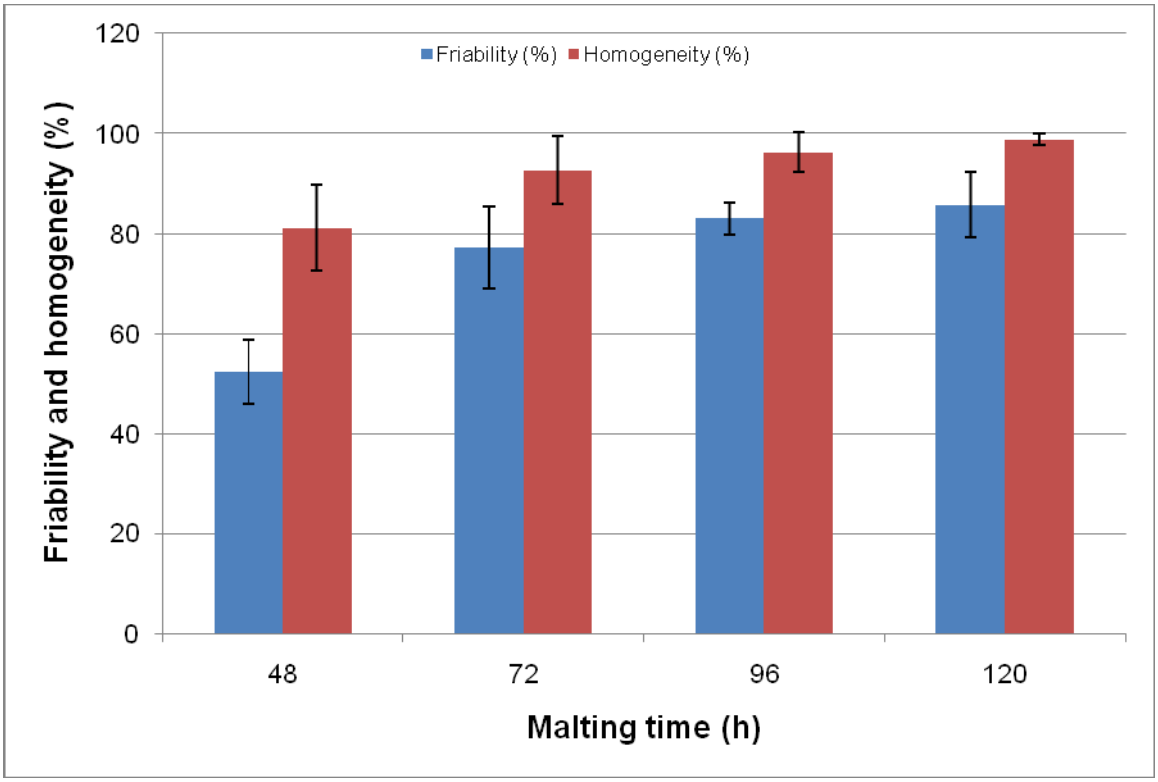


Figure 3.5: Friability and homogeneity (%) of kilned malt following steeping, during 5 d aerobic germination in the micromalting unit, n = 3 tests on the same malt ± standard deviation

3.1.5. β-Glucanase activity of malt

A positive correlation between endosperm modification, β-glucan breakdown and endo-β-glucanase development has been made. Extensive cell wall breakdown by endo-β-glucanase is an important feature of high quality malts (Palmer *et al*., 1989; MacGregor *et al*., 1993; 1994 a). β-Glucanase activity increased initially with germination

progression but, then levelled off to ~ 725 U g^{-1} following 96 h malting (Figure 3.6).

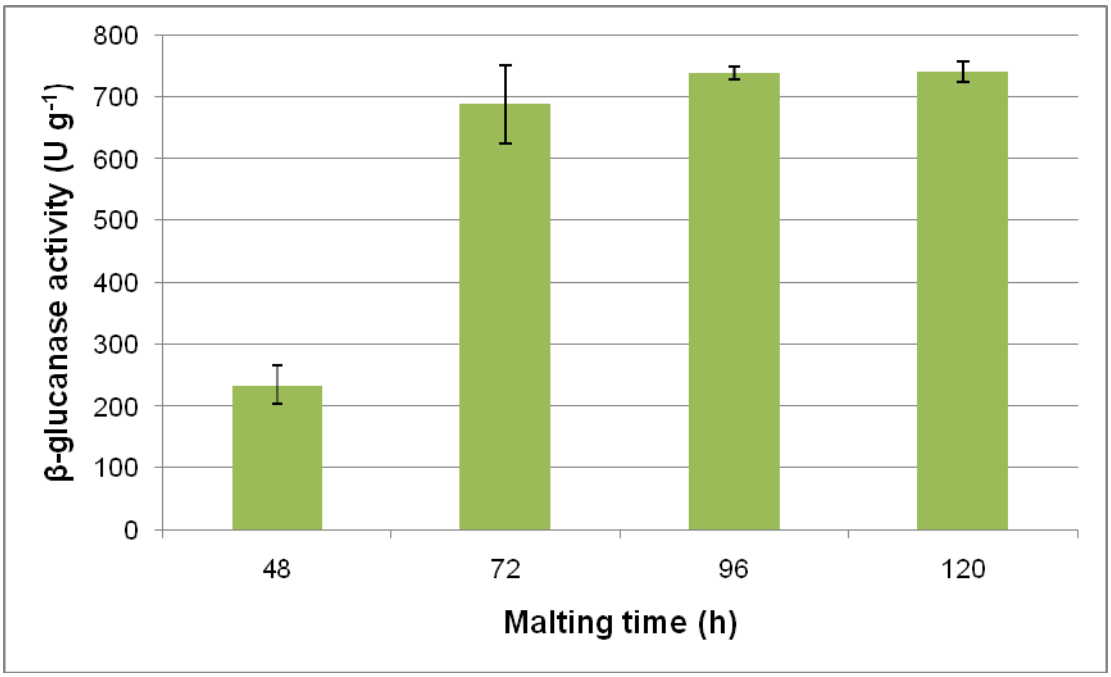


Figure 3.6: β-glucanase activity (U g^{-1}) of kilned malt following steeping, during 5 d aerobic germination in the micromalting unit, n = 3 tests on the same malt ± standard deviation

3.1.6. Wort pH

The role of pH is essential in beer production. It governs most of the physical (for example inorganic ion solubility) chemical (for example isomeration of alpha acid) and enzymatic reactions which occur and creates the necessary living environment for yeast growth. The pH of the beer contributes to the taste and character, acid is one of four principal taste sensations and will hence affect the way the consumer perceives the flavour compounds.

The typical pH for the brewing water of a standard lager is neutral (pH 7.0), as the brewing operation progresses the pH generally decreases (O'Rourke, 2002 a). A standard lager mash is generally pH 5.6 ± 0.2, boiled wort is pH 5.4 and a pH of 4.0 is attained at the end of fermentation. Wort was obtained following 1 h mashing at 65 ^{o}C as described section 2.3.6. The pH of the wort was measured using a Griffin pH meter (model 80) and compared to those expected by the Institute of Brewing for wort made from lager, ale and distillers' malt (Table 3.4). The pH of the 5 d aerobic 1 d anaerobic micromalt appears notably lower than other malt samples tested.

Wort sample	pH of wort sample
Wort made from 5 d aerobic micromalt	5.55
Wort made from 5 d commercial malt	5.6
Wort made from 6 d aerobic micromalt	5.83
Wort made from 5 d aerobic 1 d anaerobic micromalt	4.69
Wort made from IOB lager malt	5.9
Wort made from IOB ale malt	5.6
Wort made from IOB distillers' malt	5.9

Table 3.4: pH of wort derived from 5 d aerobic micromalt, 5 d commercial malt, 6 d aerobic micromalt, 5 d aerobic 1 d anaerobic micromalt as well as those expected by the Institute of Brewing for wort made from lager, ale and distillers' malt (IOB analysis taken from Palmer, 2006)

3.1.7. Wort hot water extract

Extract is an indication of the amount of sugar recovered from the malt after mashing. The hot water extract was only measured in wort made from the aerobic micromalt from 2 d to 5 d as the 6 d aerobic micromalt and the 5 d aerobic 1 d anaerobic micromalt derived from this sample and it was assumed that the wort made from 5 d commercial malt was of industrial quality. The hot water extract increased steadily from ~273 $l^{o}kg^{-1}$ at 48 h malting to ~289 $l^{o}kg^{-1}$ at 120 h malting (Figure 3.7), this value was slightly lower than is specified by the IOB for lager and ale malts (Table 3.5) and higher than required for distilling malts, but the measurements show a relatively large standard deviation.

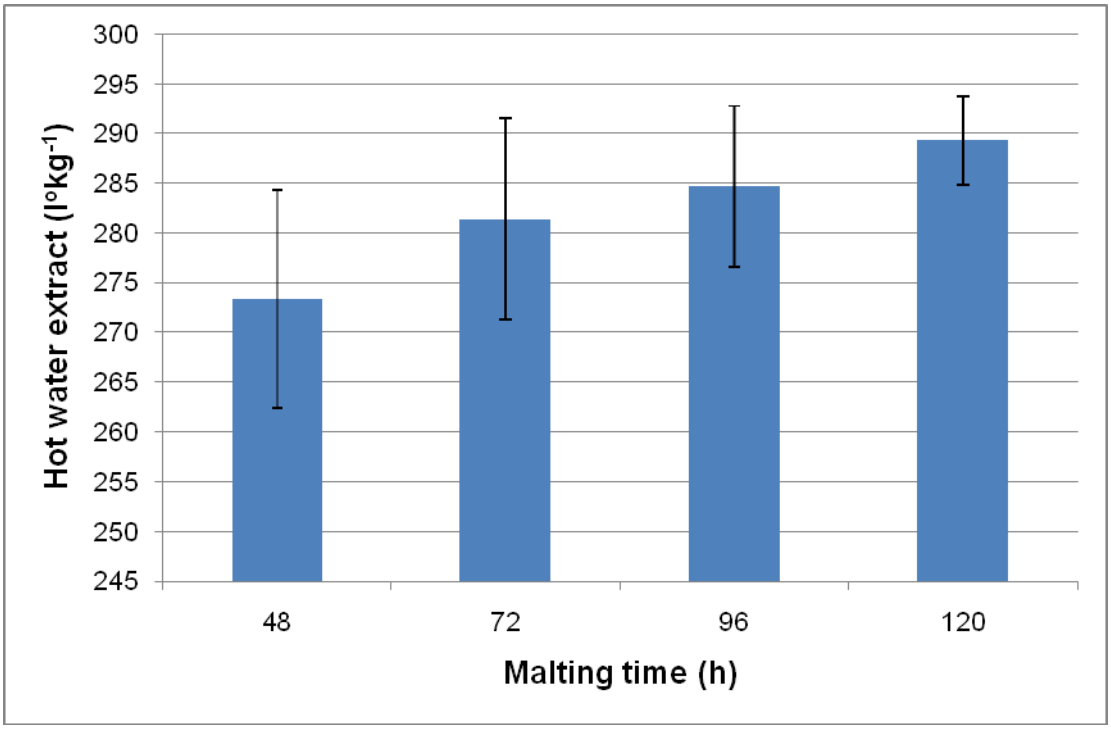


Figure 3.7: Hot water extract ($l^{o}kg^{-1}$) of kilned malt during 5 d aerobic germination in the micromalting unit, n = 3 tests on the same malt ± standard deviation

Wort sample	Hot water extract ($l^{o}kg^{-1}$)
Wort made from IOB lager malt	300 - 315
Wort made from IOB ale malt	305 - 315
Wort made from IOB distillers' malt	300 - 310

Table 3.5: **Hot water extract (lokg-1) expected by the Institute of Brewing for wort made from lager, ale and distillers' malt made from standard 2-row barley (IOB analysis taken from Palmer, 2006 and Bathgate, 1989)**

3.2. Malt and wort carbohydrate composition

The starch and sugar composition of a malt or wort gives an insight into the nutritional properties it has for yeast during fermentation.

3.2.1. Total starch content of malt

During 5 d (120 h) aerobic malting *Optic* grains were removed from the micromalting unit, kilned to 4.5 % (w/w) moisture, ground and the total starch content was

determined using the amyloglucosidase/α-amylase assay procedure from Megazyme (described in section 2.4.1). The total starch decreased by ~ 22 % (Figure 3.8) from 48 h to 120 h malting. This decrease in starch content is typically observed during malting.

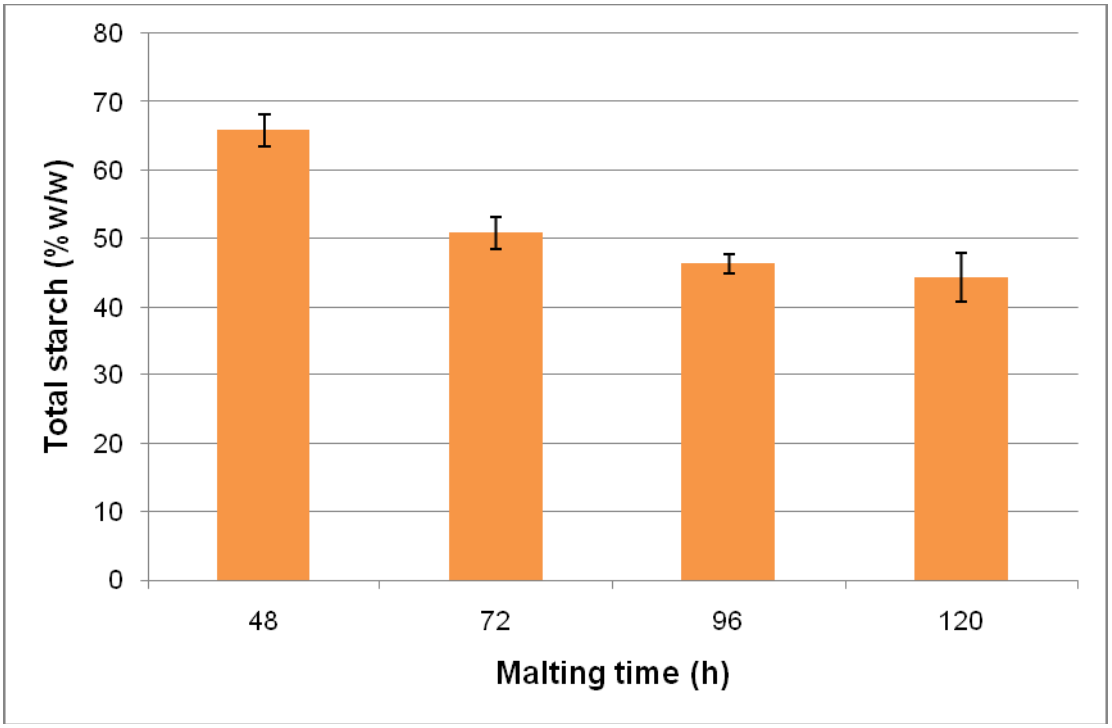


Figure 3.8: Total starch content (% w/w) of kilned malt during 5 d aerobic germination in the micromalting unit, n = 3 tests on the same malt ± standard deviation

3.2.2. Fermentable sugar content of wort

The standard method of analysing wort fermentability is called the apparent attenuation limit (AAL) and is calculated using measurements taken before and after fermentation. This standard method is however time consuming and involves using large volumes of wort; it is therefore unsuitable for the large number of sample used in this study. As an alternative high performance liquid chromatography (HPLC) was used to determine the levels of glucose, fructose, sucrose, maltose and maltotriose in wort made from malt flour during 5 d aerobic micromalting and ground single grains following 5 d aerobic micromalting, 5 d aerobic commercial malting, 6 d aerobic micromalting and 5 d aerobic 1 d anaerobic micromalting (see section 2.4.2).

Wort produced from 48 to 120 h aerobic micromalting had a total fermentable sugar level increase of ~ 37000 mg l^{-1} (Figure 3.9). This increase can be attributed to an

increase of ~ 40000 mg l^{-1} in maltose (Figure 3.10), the glucose level was found to decrease from ~ 26000 mg l^{-1} to ~ 6000 mg l^{-1} between 72 and 120 h micromalting.

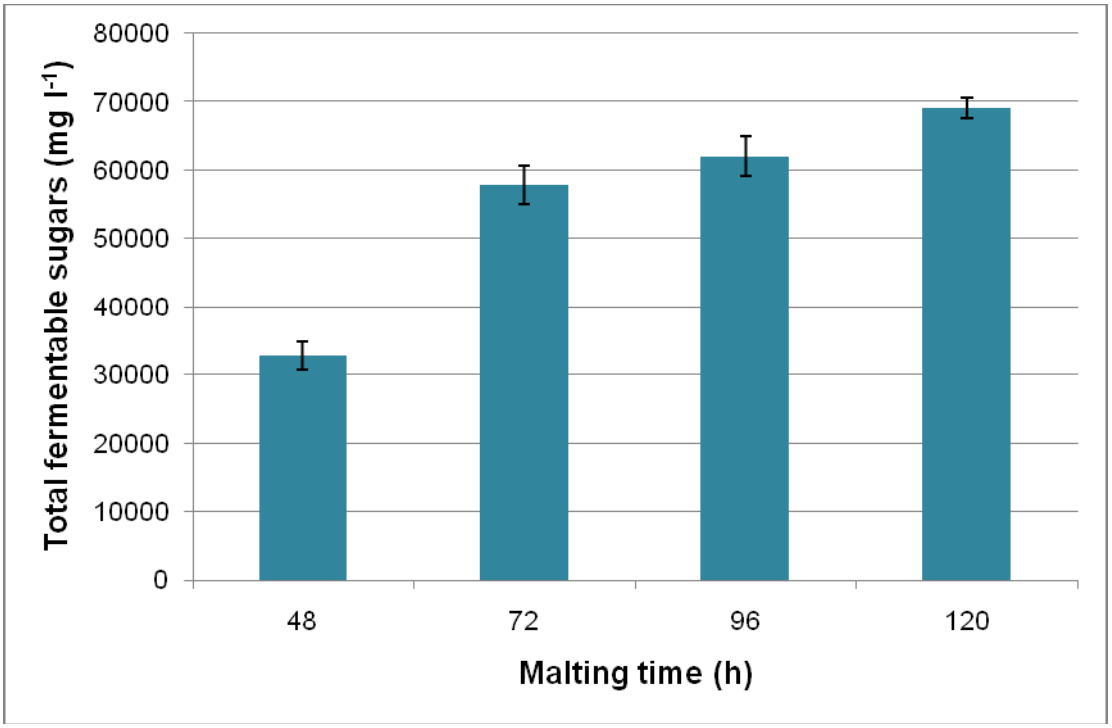


Figure 3.9: Total fermentable sugar content (mg l^{-1}) of wort made by mashing kilned malt during 5 d aerobic germination in the micromalting unit, n = 3 tests on the same malt ± standard deviation

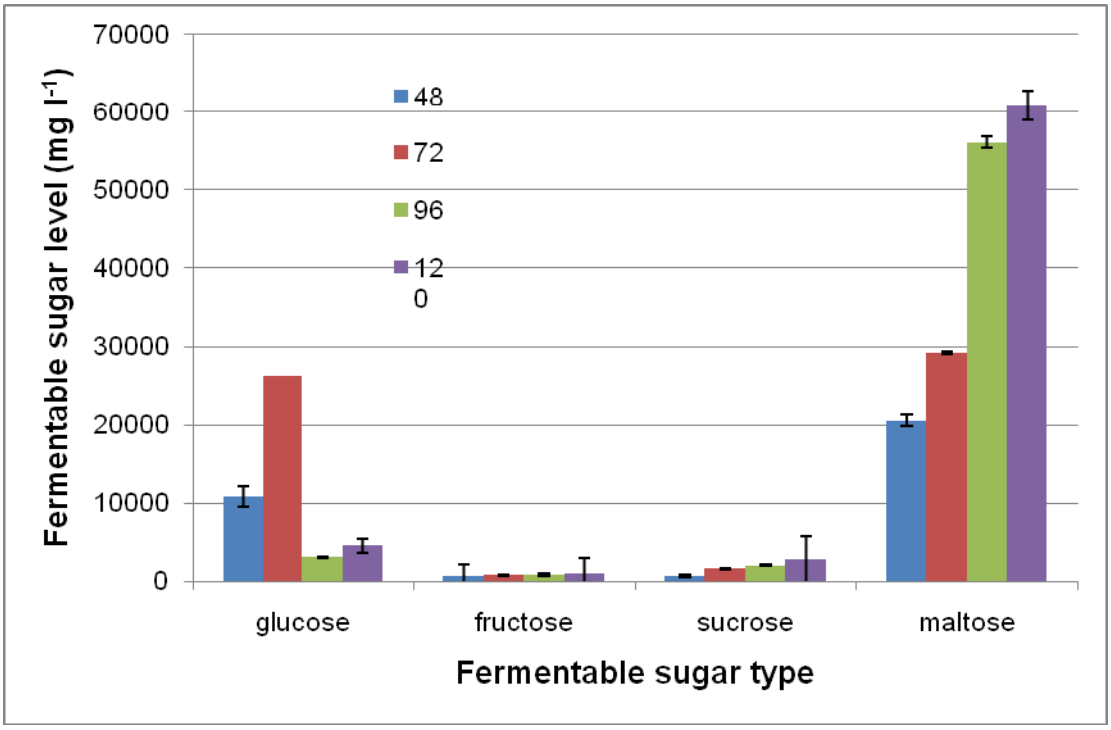


Figure 3.10: Glucose, fructose, sucrose and maltose content (mg l^{-1}) of wort made by mashing kilned malt during 5 d aerobic germination in the micromalting unit, n = 3 tests on the same malt ± standard deviation

Fermentable sugar levels in wort produced by ground single grains of 5 d aerobic micromalt and 5 d aerobic commercially produced malt are shown in Figure 3.11and for 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt in Figure 3.12.

Statistical analysis for all malt types are shown in Table 3.6. The mean single grain fermentable sugar content was highest in 5 d aerobic commercially produced malt. The 5 d aerobic micromalt had the lowest mean and despite similar ranges of data single grains of 5 d aerobic micromalt were consistently lower than those obtained for the commercial malt. The second highest mean was obtained by the 5 d aerobic 1 d anaerobic micromalt. The greatest standard deviation was observed for values of the 6 d aerobic micromalt.

Increased levels of glucose, maltose and maltotriose are responsible for high levels of fermentable sugars in the commercial malt and 5 d aerobic 1 d anaerobic micromalt (Figure 3.13). When wort fermentable sugar content (mg l^{-1}) were calculated from single grain (mg $grain^{-1}$) and single grain weight (g) data it was discovered that the highest calculated level of fermentable sugar was present in the 5 d aerobic 1 d

anaerobic micromalt (Figure 3.14), this was followed by the 5 d aerobic commercially produced malt. Lowest levels of fermentable sugars were found in the 5 d aerobic micromalt.

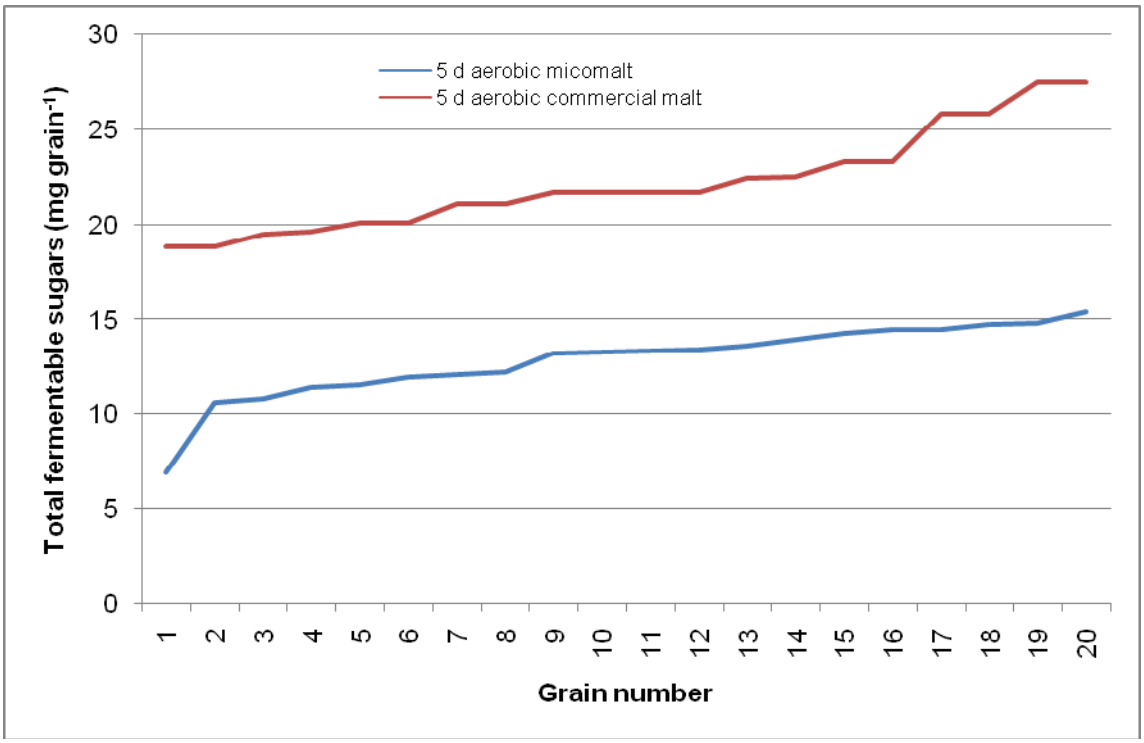


Figure 3.11: Total fermentable sugars (mg grain^{-1}) of wort obtained from 20 single kilned malt grains produced following 5 d aerobic micromalting unit at Heriot-Watt University or commercially produced at Simpsons Maltings

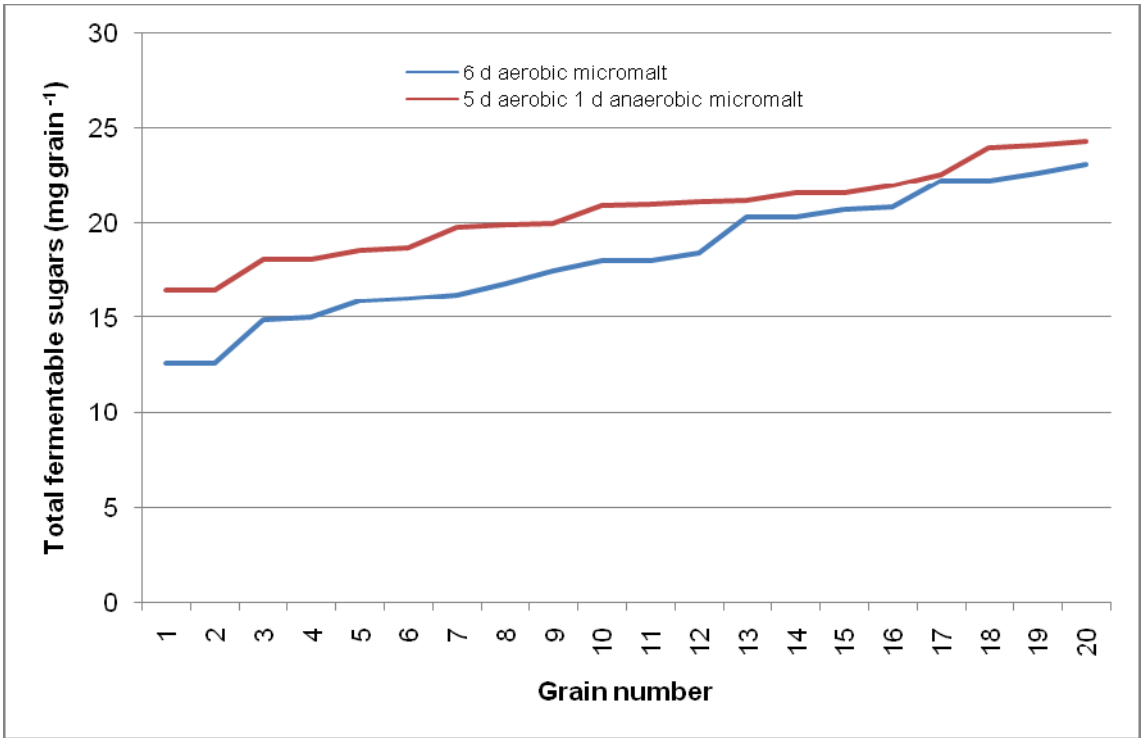


Figure 3.12: Total fermentable sugars (mg grain^{-1}) of wort obtained from 20 single kilned malt grains produced following 6 d aerobic germination in the micromalting unit at Heriot-Watt University or 5 d aerobic micromalting and 1 d anaerobic germination

Statistic	5 d aerobic micromalt	5 d aerobic commercial malt	6 d aerobic micromalt	5 d aerobic 1 d anaerobic micromalt
Mean	12.81	22.2	18.21	20.53
Standard deviation	1.97	2.66	3.23	2.31
Median	13.32	21.7	18	20.95
Range	8.47	8.7	10.5	7.8
% cv	15.41	11.97	17.72	11.25

Table 3.6: Statistical analysis for fermentable sugar content of wort (mg grain^{-1}) made from 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt

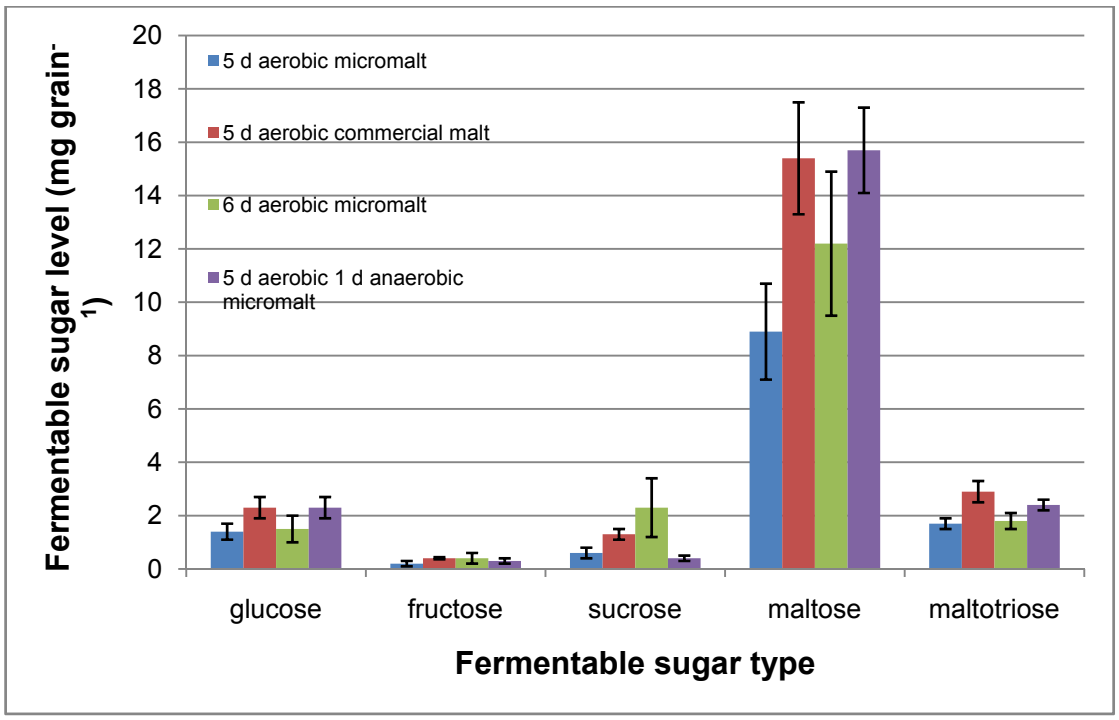


Figure 3.13: Average glucose, fructose, sucrose, maltose and maltotriose content (mg grain^{-1}) of wort obtained from single kilned malt grains produced following 5 and 6 d aerobic micromalting, 5 d aerobic 1 d anaerobic micromalting and 5 d aerobic commercial malting, n = average of 20 single grains ± standard deviation

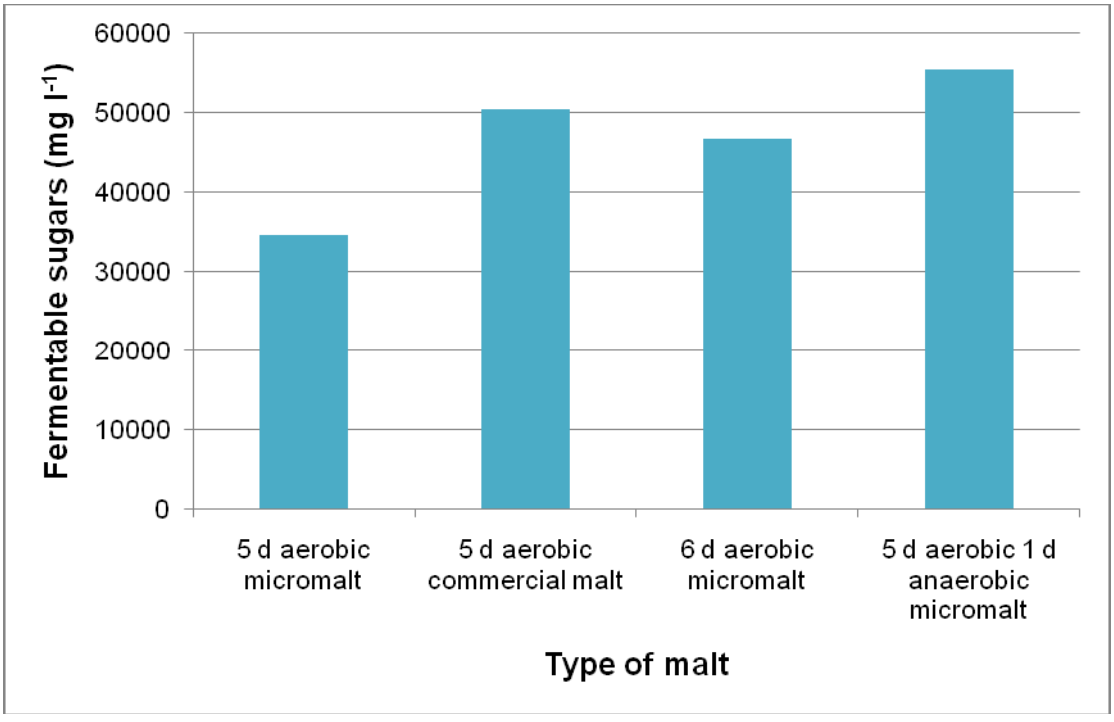


Figure 3.14: Calculated fermentable sugar content of 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic malt and 5 d anaerobic 1 d anaerobic micromalt calculated using mean fermentable sugar content (mg grain^{-1}) values (Table 3.6) and mean single grain weight (g) values (Figure 3.2), where 10 g flour is mashed as a slurry in 100 ml water

3.3. Nitrogen content of wort

The nitrogenous constituents of wort include amino acids, peptides, polypeptides, proteins, nucleic acids and their degradation products. Some nitrogenous constituents are essential factors for yeast growth and replication during fermentation and wort must contain an adequate supply. Most proteins are not assimilated by the yeast during fermentation but, can be precipitated which causes them to adhere to the yeast. Excessive levels of protein in the wort can lead to fining problems.

3.3.1. Total soluble nitrogen content of the wort

Levels of total soluble nitrogen in the wort commercially fall within the range 600 to 900 mg l^{-1} (O'Rourke, 2002 a).

Total soluble nitrogen levels at 120 h micromalting were ~ 650 mg l^{-1} which is commercially acceptable (Figure 3.15).

About 80 % of single grains malted for 5 d aerobically in a commercial maltings contained higher levels of total soluble nitrogen than single grains micromalted aerobically for 5 d (Figure 3.16).

Six d aerobic micromalted grains produced higher total soluble nitrogen than 5 d aerobic 1 d anaerobic micromalted grains (Figure 3.17).

Statistical analysis (Table 3.7) revealed the highest mean total soluble nitrogen level and standard deviation to be found in the 5 d aerobic commercially produced grains, while lowest mean and standard deviation was obtained by the 5 d aerobic 1 d anaerobically micromalted grains.

The cumulative distribution curve (Figure 3.18) reflect the pattern of total soluble nitrogen by all malt samples, the grains of the 5 d aerobic commercial malt had significantly higher level of total soluble nitrogen in comparison to other malt samples.

The high levels of total soluble nitrogen in 5 d aerobic commercially produced malt are mirrored in the calculated analysis of total soluble nitrogen (mg l^{-1}) in wort (Figure 3.19). Interestingly the total soluble nitrogen content of wort calculated theoretically using the mean of 5 d aerobic micromalt grains and 5 d aerobic 1 d anaerobic micromalt grains would not meet industrial specifications while, the mean 5 d aerobic commercial grain would have a total soluble nitrogen level far exceeding these specifications.

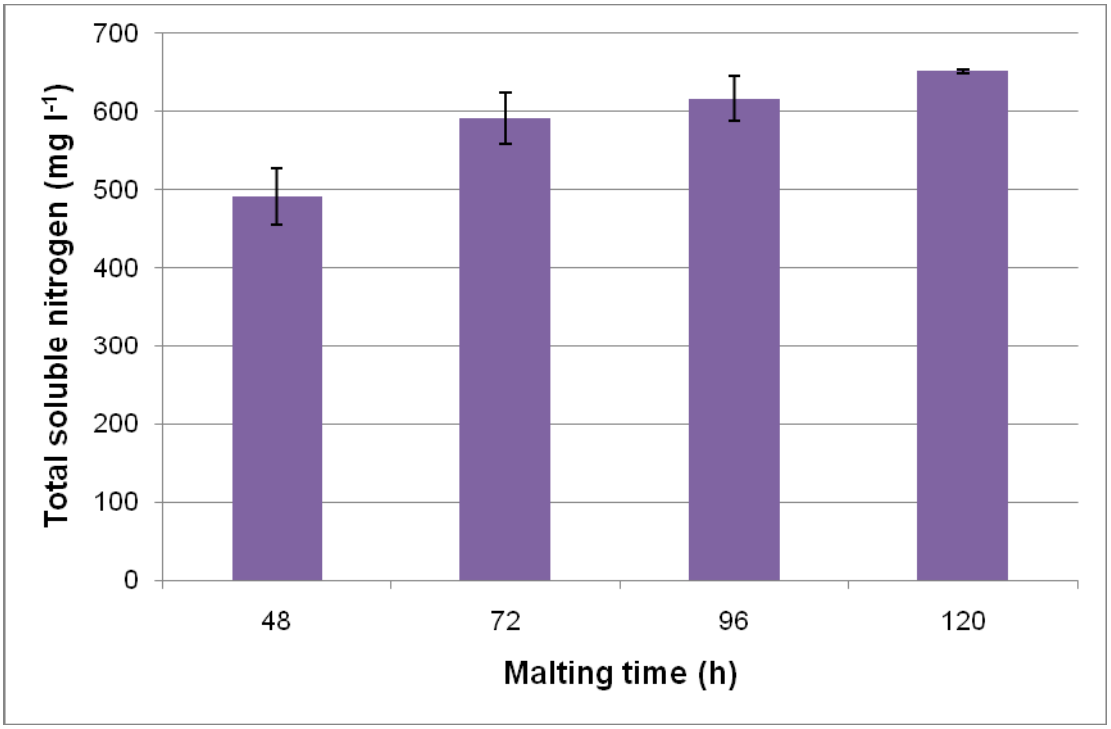


Figure 3.15: Total soluble nitrogen content (mg l^{-1}) of wort made by mashing kilned malt during 5 d aerobic germination in the micromalting unit, n = 3 tests on the same malt ± standard deviation

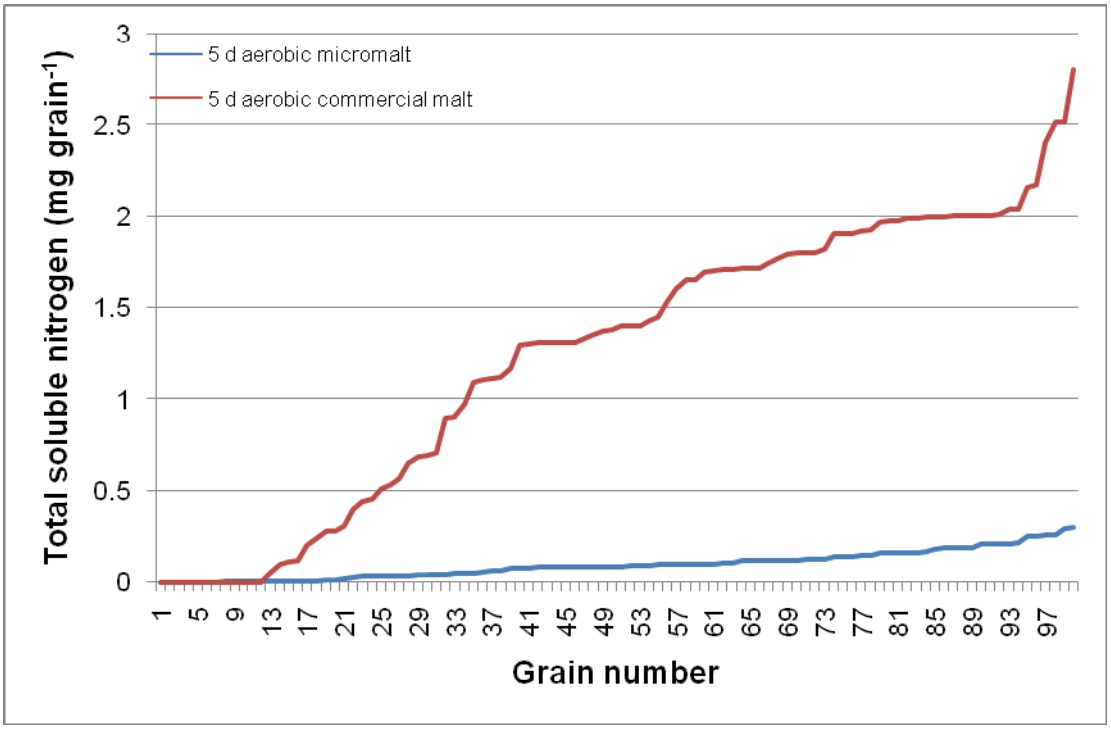


Figure 3.16: Total soluble nitrogen content (mg grain^{-1}) of wort obtained from 100 single kilned malt grains produced following 5 d aerobic micromalting and 5 d aerobic commercial malting

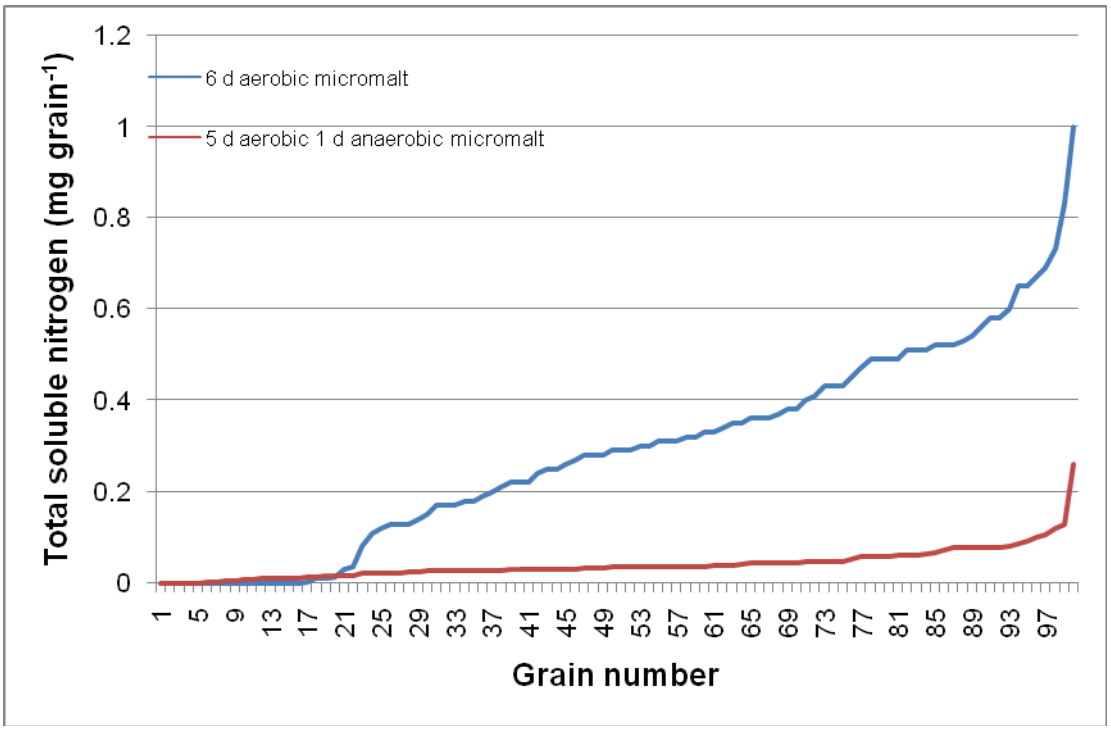


Figure 3.17: Total soluble nitrogen content (mg grain^{-1}) of wort obtained from 100 single kilned malt grains produced following 6 d aerobic micromalting and 5 d aerobic, 1 d anaerobic micromalting

Statistic	5 d aerobic micromalt	5 d aerobic commercial malt	6 d aerobic micromalt	5 d aerobic 1 d anaerobic micromalt
Mean	0.097	1.243	0.29	0.041
Standard deviation	0.074	0.767	0.22	0.035
Median	0.087	1.391	0.29	0.036
Range	0.298	2.8	1	0.26
%cv	76.199	61.651	76.033	83.652

Table 3.7: Statistical analysis for total soluble nitrogen content of wort (mg grain^{-1}) made from 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt

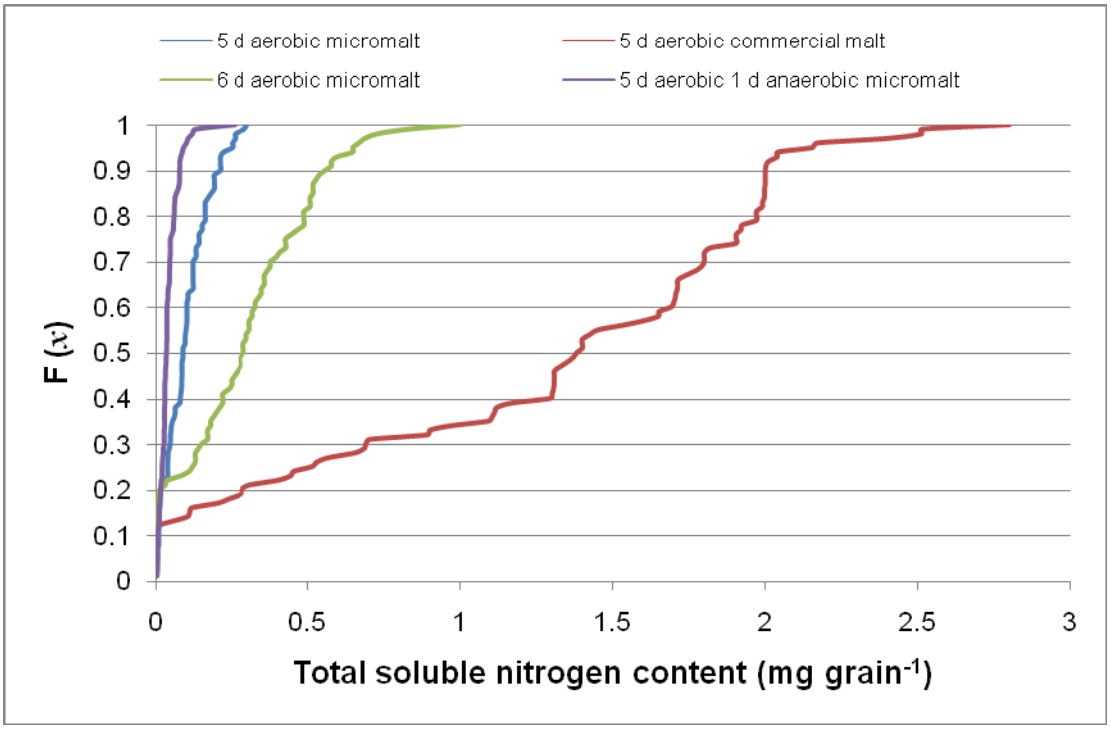


Figure 3.18: Cumulative distribution function (CDF) of total soluble nitrogen (mg grain^{-1}) for 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt

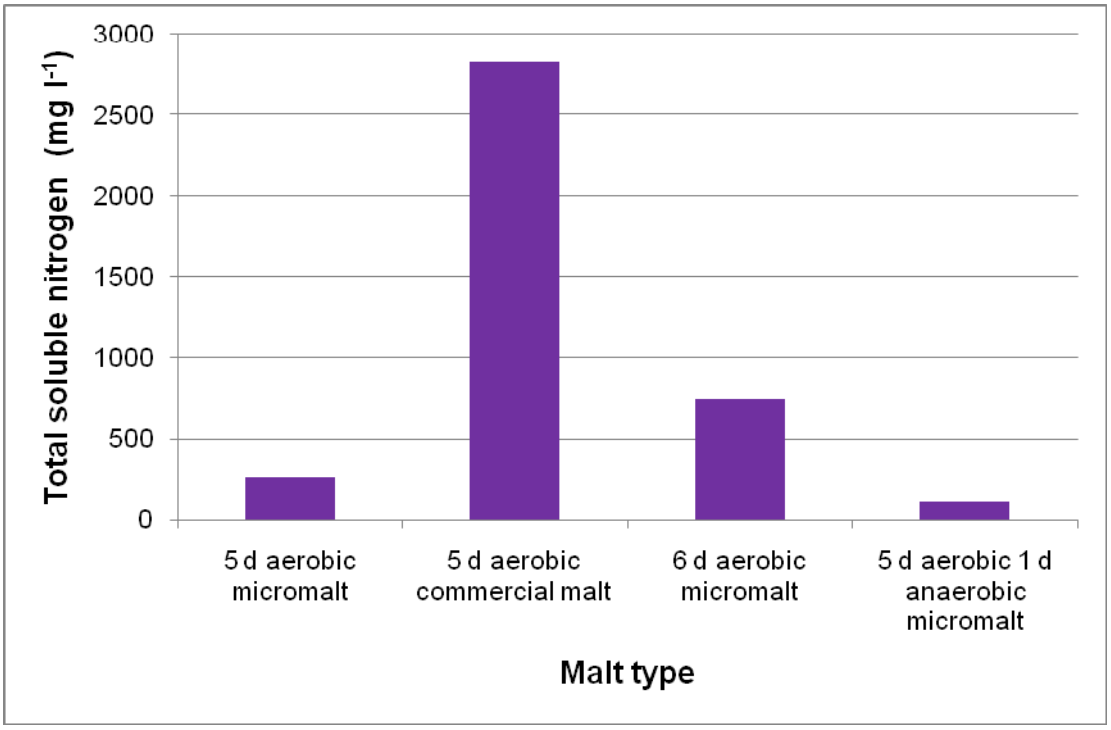


Figure 3.19: Total soluble nitrogen content of 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic malt and 5 d anaerobic 1 d anaerobic micromalt when calculated using mean total soluble nitrogen content (mg grain^{-1}) values (Table 3.7) and mean single grain weight (g) values (Table 3.2), 10 g flour would normally be mashed as a slurry in 100 ml water

3.3.2. Free α-amino nitrogen content of the wort

Commercially viable wort usually contains free amino nitrogen values of 160 to 190 mg l $^{-1}$ (O'Rourke, 2002 a). Lower levels of free amino nitrogen can cause problems with sticky fermentations but, high free amino nitrogen constituents in wort are prone to infection by micro-organisms.

Wort produced from malt flour contained ~ 135 mg l^{-1} free amino nitrogen, this is less than industrially specified but, the standard deviation was high (Figure 3.20).

Free amino nitrogen values of 5 d aerobic single grains (mg grain^{-1}) produced via micromalting and commercial malting are shown in Figure 3.21.

Values of free amino nitrogen in single grains produced by 6 d aerobic and 5 d aerobic 1 d anaerobic micromalting were similar (Figure 3.22) with a few of the 5 d aerobic 1 d anaerobic grains having high values.

The highest mean free amino nitrogen and standard deviation are achieved by the 5 d aerobic commercially produced malt (Table 3.8). The lowest mean value and standard deviation for free amino nitrogen was found when the 5 d aerobically micromalted grains were analysed.

The cumulative distribution curve (Figure 3.23) shows similar patterns of free amino nitrogen values for the 5 d aerobic commercially produced malt grains, the 6 d aerobic micromalt and the 5 d aerobic 1 d anaerobic micromalt, with the exception of a few 5 d commercial malt grains with high values. The 5 d aerobic micromalt exhibits much lower levels of free amino nitrogen.

When wort free amino nitrogen (mg l^{-1}) levels were calculated the 5 d aerobic 1 d anaerobic micromalt produced the highest value, levels were however ~ 60 mg l^{-1} lower than is deemed commercially viable (Figure 3.24).

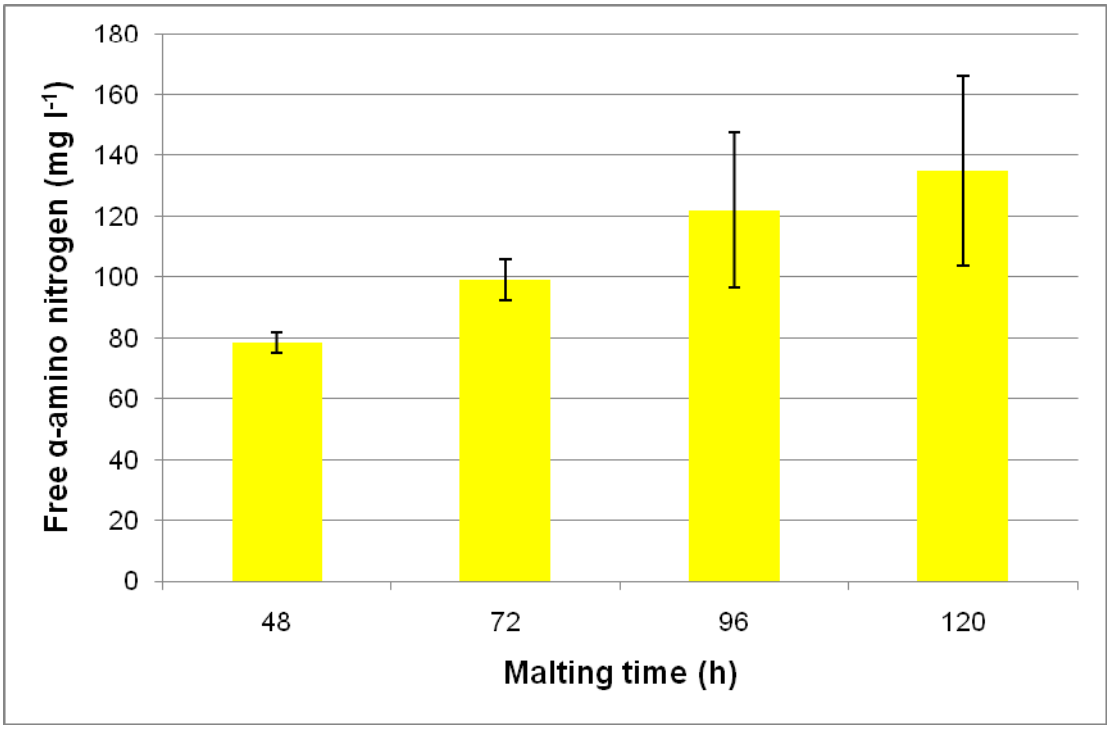


Figure 3.20: Free α-amino nitrogen content (mg l^{-1}) of wort made by mashing kilned malt during 5 d aerobic germination in the micromalting unit, n = 3 tests on the same malt ± standard deviation

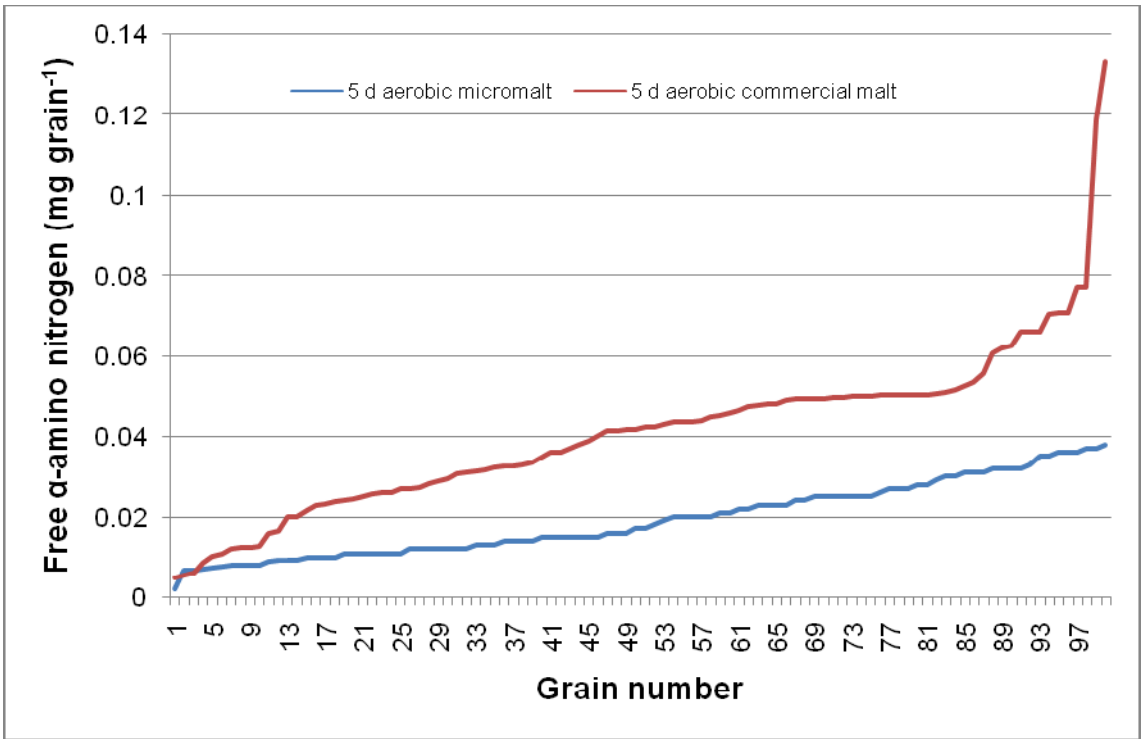


Figure 3.21: Free α-amino nitrogen content (mg $grain^{-1}$) of wort obtained from 100 single kilned malt grains produced following 5 d aerobic germination either in the micromalting unit at Heriot-Watt University or commercially produced at Simpsons Maltings

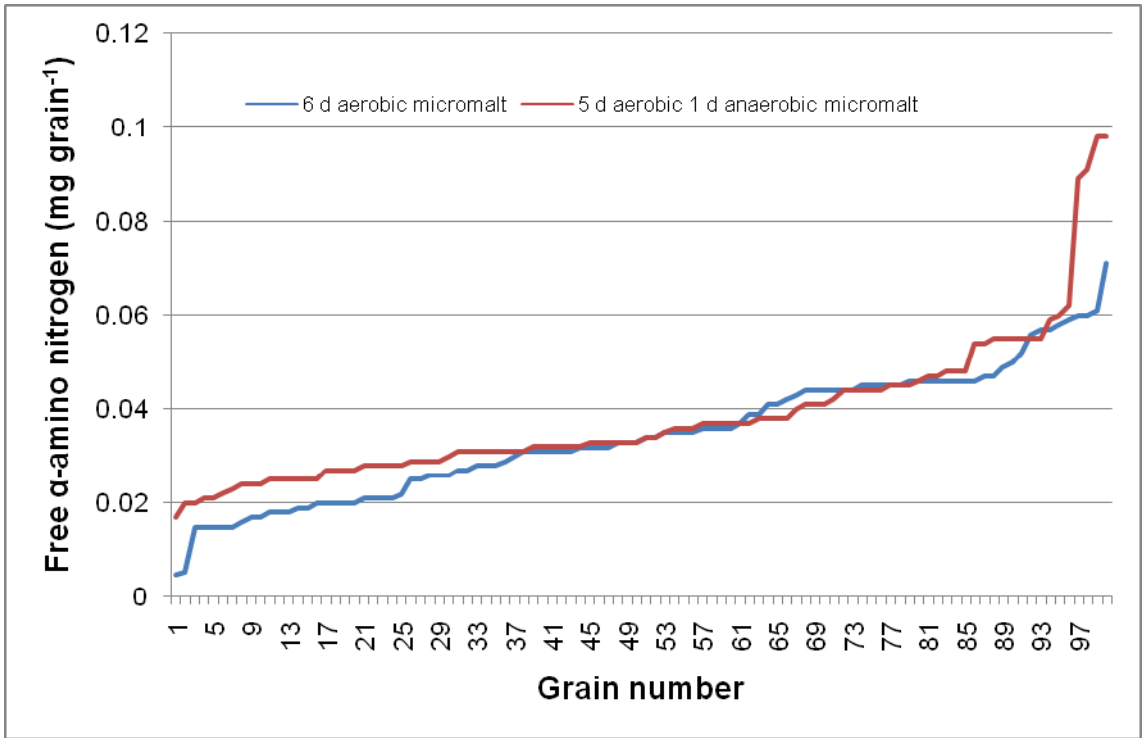


Figure 3.22: Free α-amino nitrogen content (mg grain^{-1}) of wort obtained from 100 single kilned malt grains produced following 6 d aerobic germination in the micromalting unit at Heriot-Watt University or 5 d aerobic micromalting and 1 d anaerobic germination

Statistic	5 d aerobic micromalt	5 d aerobic commercial malt	6 d aerobic micromalt	5 d aerobic 1 d anaerobic micromalt
Mean	0.019	0.041	0.034	0.038
Standard deviation	0.009	0.021	0.0135	0.015
Median	0.017	0.042	0.034	0.034
Range	0.036	0.128	0.066	0.081
%cv	47.217	50.289	39.188	40.045

Table 3.8: Statistical analysis for free α-amino nitrogen content of wort (mg grain-1) made from 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt

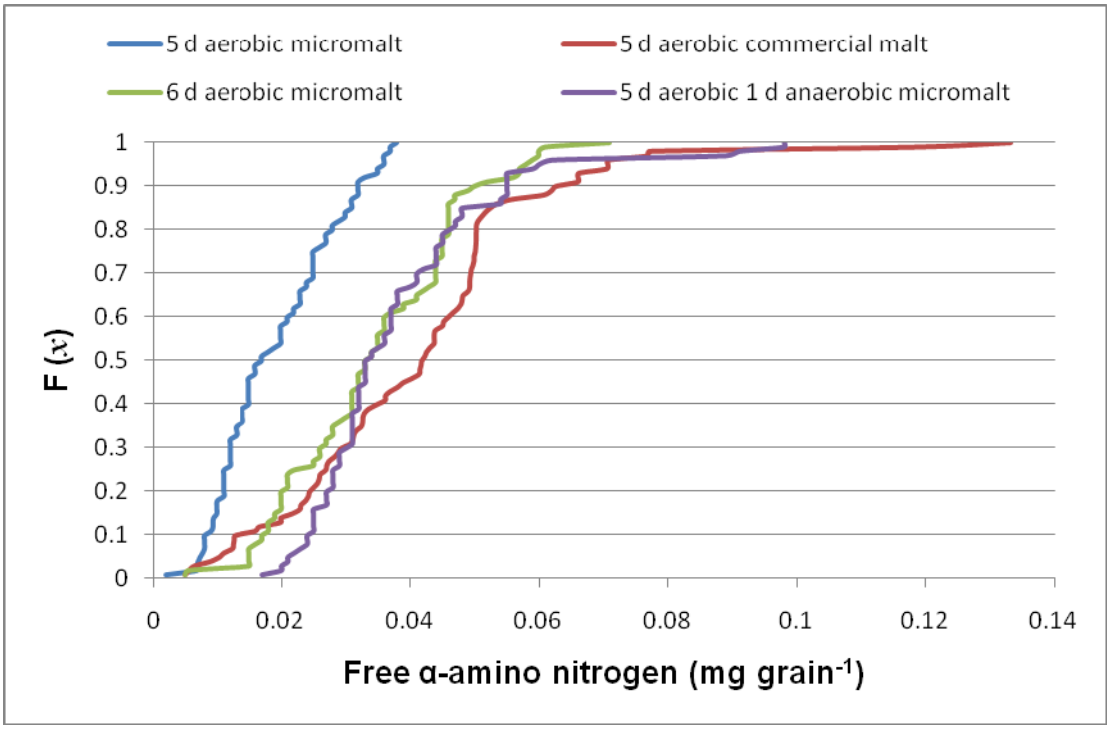


Figure 3.23: Cumulative distribution function (CDF) of free α-amino nitrogen (mg grain^{-1}) for 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt

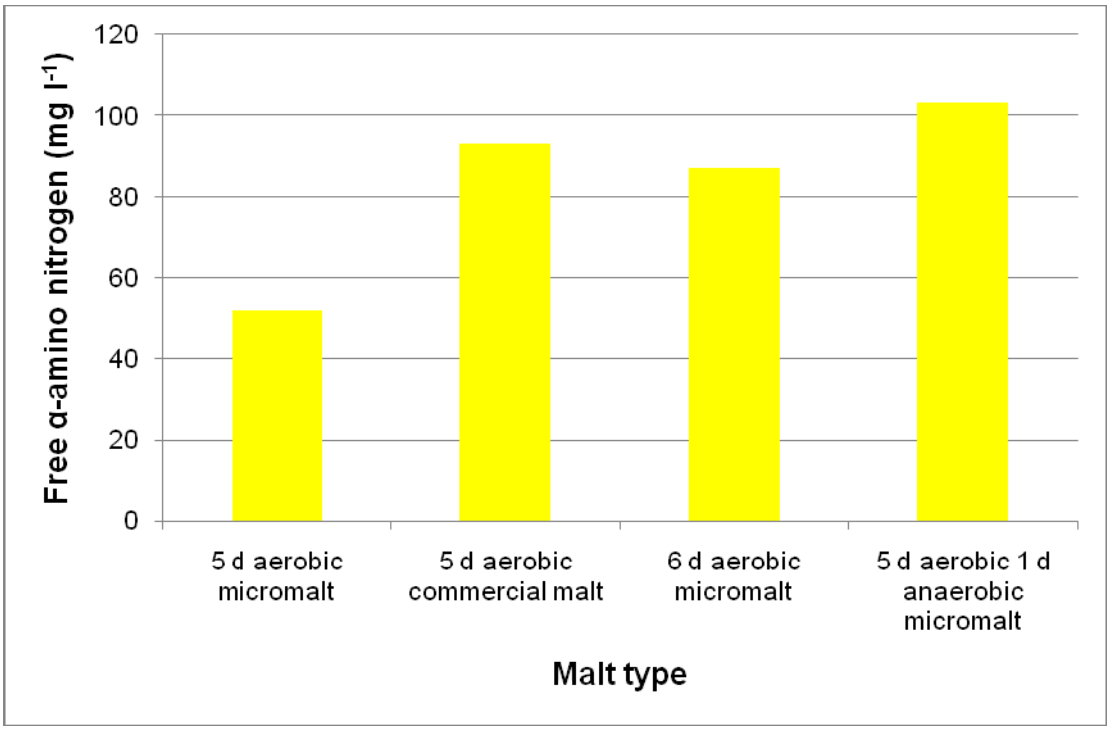


Figure 3.24: Free α-amino nitrogen content of 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic malt and 5 d anaerobic 1 d anaerobic micromalt when calculated using mean free α-amino nitrogen content (mg grain^{-1}) values (Table 3.8) and mean single grain weight (g) values (Table 3.2), 10 g flour is normally mashed as a slurry in 100 ml water

3.4. Enzyme activity analysis

3.4.1. Malt α-amylase

α-amylase plays a predominant role in the initial stages of amylolytic breakdown of reserve carbohydrates in starchy barley grains during germination.

mRNA was extracted from barley grains during development and germination, blotted and probed specifically for α-amylase type A mRNA (encoding isozymes AMY1 and AMY2). Northern blots (Figures 3.25 and 3.26) show α-amylase transcript to be present at 1 week post anthesis; its expression decreased at this time, there does not appear to be any α-amylase mRNA at 5 weeks post anthesis (Figure 3.25). α-amylase is potentially expressed throughout germination (Figure 3.26).

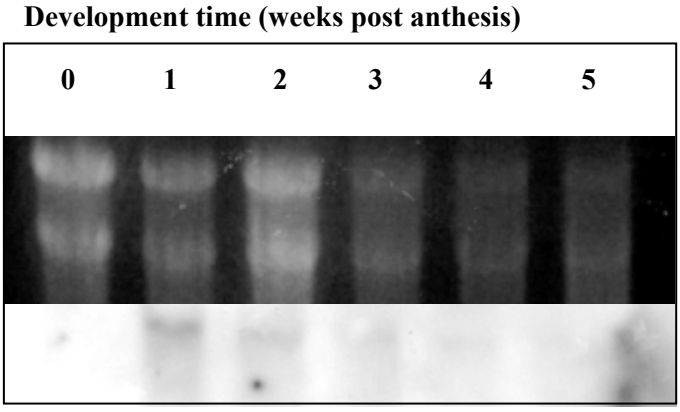


Figure 3.25: Northern blot analysis of α-amylase type A gene expression during grain development

Top: Ethidium bromide stained RNA

Bottom: Chemiluminescent detection of hybridised α-amylase RNA probe.

0 is at time of anthesis.

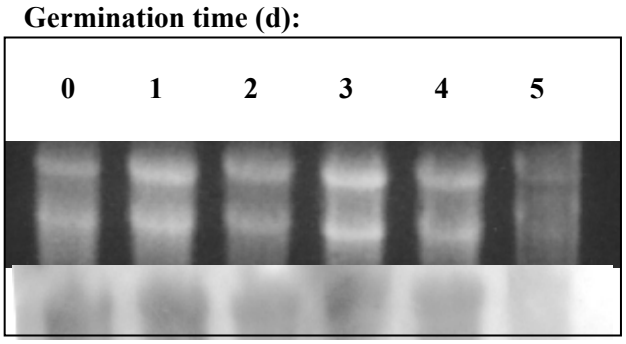


Figure 3.26: Northern blot analysis of α-amylase type A gene expression during grain germination

Top: Ethidium bromide stained RNA

Bottom: Chemiluminescent detection of hybridised α-amylase RNA probe.

0 is at time of maturation.

α-Amylase activity (mU g^{-1}) increased throughout malting (Figure 3.27). Figure 3.28 shows α-amylase activity of 100 single grains which were either micromalted or commercially malted for 5 d aerobically.

Figure 3.29 shows that all 100 single grains from the 5 d aerobic 1 d anaerobic micromalt contained higher α-amylase activity than grains from the 6 d aerobic micromalt.

Interestingly the 5 d aerobic commercially produced malt had the lowest mean α-amylase activity and the highest standard deviation while the 5 d aerobic 1 d anaerobic malt had the highest α-amylase activity and lowest standard deviation (Table 3.9).

The cumulative distribution curve illustrates the substantial α-amylase activity of the 5 d aerobic 1 d anaerobic micromalt grains in comparison to α-amylase activity of other malt grains (Figure 3.30).

When the α-amylase activity was calculated from the mean single grain activity the 5 d aerobic 1 d anaerobic malt had an activity that was ~25 mU g^{-1} lower than activity measured in malt flour following 120 h malting (Figure 3.27), the other malt samples had activities much lower in value.

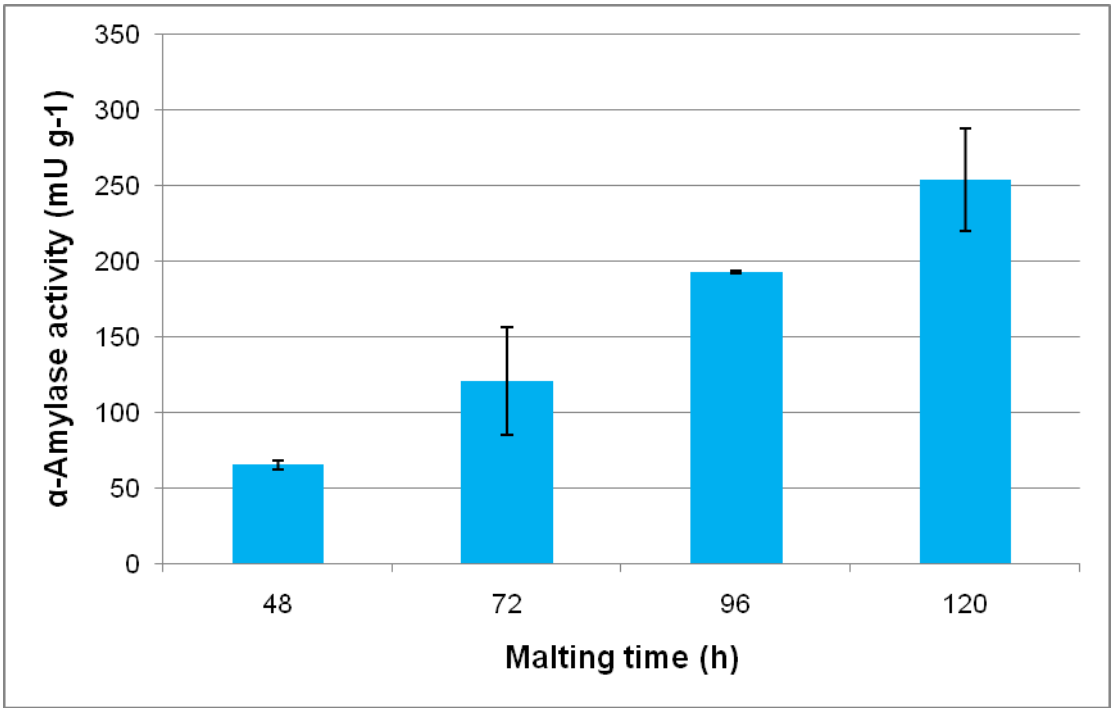


Figure 3.27: α-amylase activity (mU g^{-1}) of kilned malt during 5 d aerobic germination in the micromalting unit, n = 3 tests on the same malt ± standard deviation

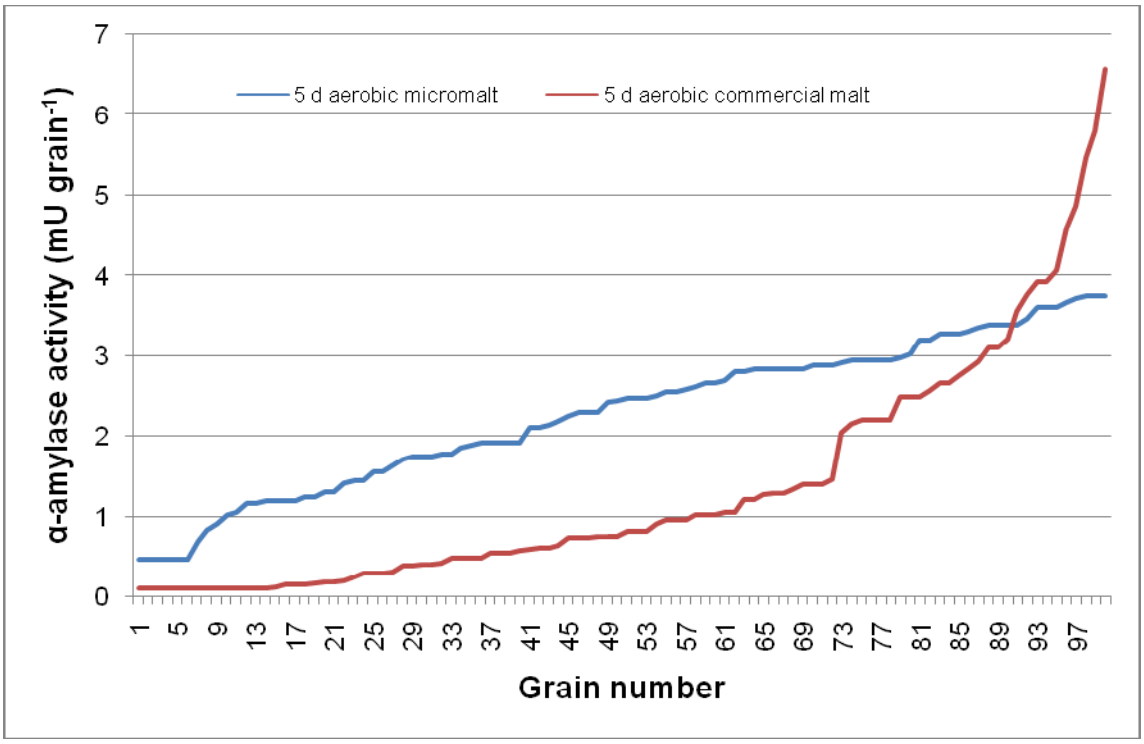


Figure 3.28: α-amylase activity (mU $grain^{-1}$) of 100 single kilned malt grains produced following 5 d aerobic micromalting at Heriot-Watt University micromalting unit and following 5 d aerobic commercial malting at Simpsons Maltings, Berwick-Upon-Tweed

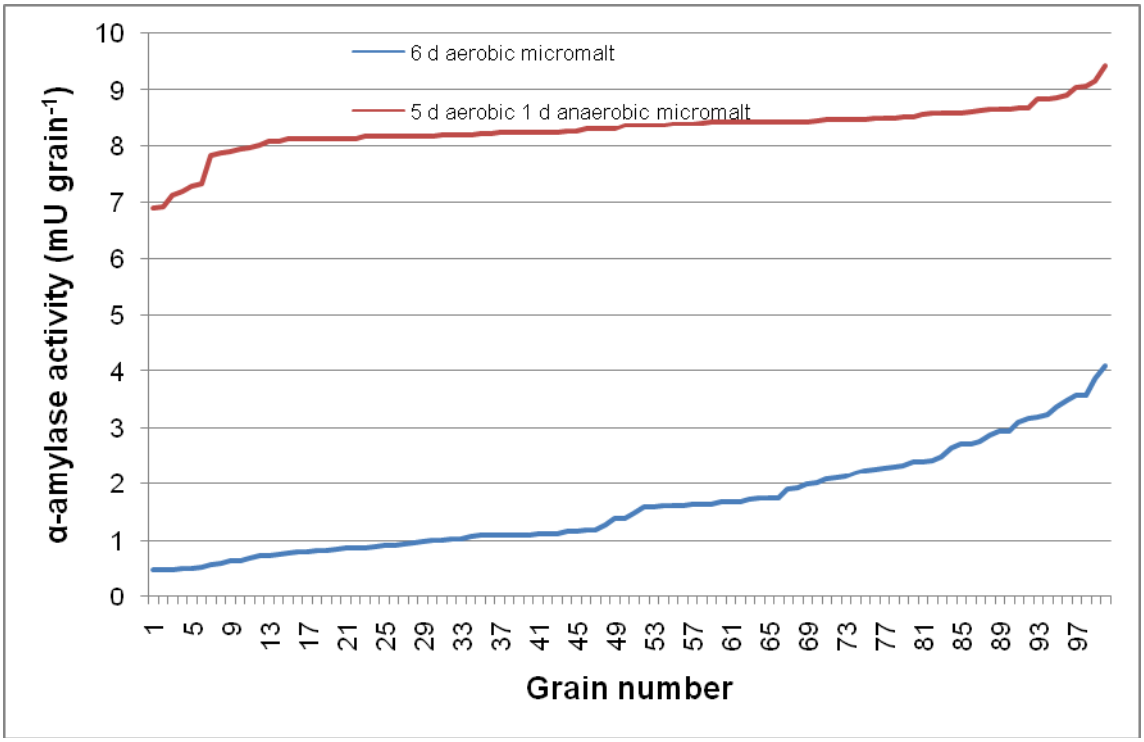


Figure 3.29: α-amylase activity (mU grain^{-1}) of 100 single kilned malt grains produced following 6 d aerobic micromalting at Heriot-Watt University micromalting unit and 5 d aerobic micromalting followed by 1 d anaerobic incubation

Statistic	5 d aerobic micromalt	5 d aerobic commercial malt	6 d aerobic micromalt	5 d aerobic 1 d anaerobic micromalt
Mean	2.28	1.35	1.63	8.32
Standard deviation	0.92	1.43	0.9	0.41
Median	2.46	0.79	1.43	8.37
Range	3.28	6.46	3.6	2.53
% cv	40.35	205.72	54.86	4.91

Table 3.9: Statistical analysis for single grain α-amylase activity (mU grain^{-1}) of 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt. All micromalt was produced at the micromaltings at Heriot-Watt University, commercial malt was produced at Simpson's maltings, Berwick-Upon-Tweed

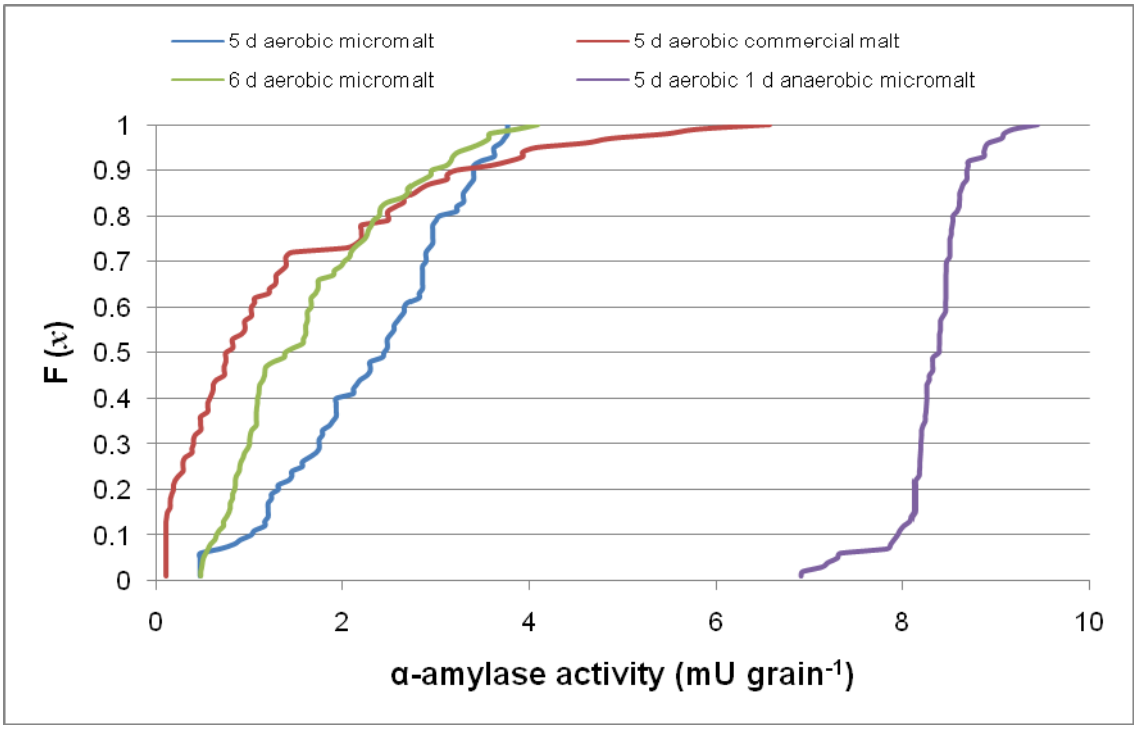


Figure 3.30: Cumulative distribution function (CDF) of α-amylase (mU grain^{-1}) for 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt

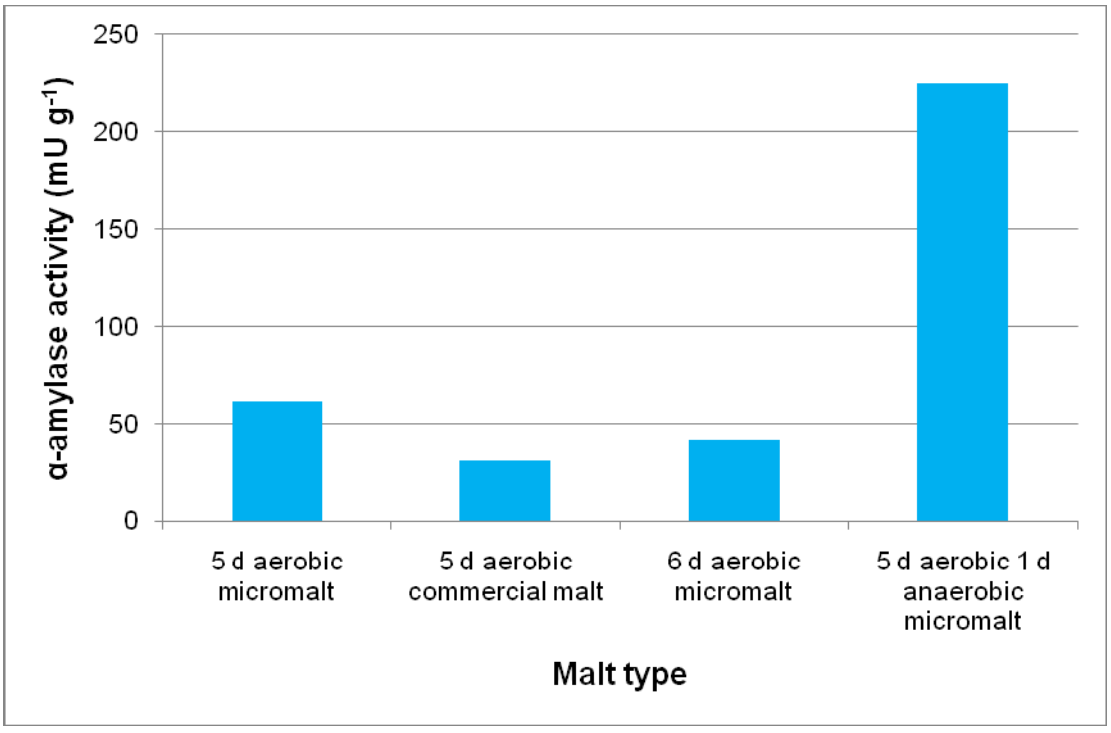


Figure 3.31: α-amylase activity (mU g^{-1}) of 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic malt and 5 d anaerobic 1 d anaerobic micromalt when calculated using mean α-amylase activity (mU grain^{-1}) values (Table 3.9) and mean single grain weight (g) values (Table 3.2)

3.4.2. β-Amylase

β-amylase is consistently considered an indicator of the capacity of malt to degrade starch to fermentable sugars. β-amylase present in mature, ungerminated grain is a measure of the suitability of the grain for brewing processes (Gibson *et al.*, 1995). β-amylase activity assayed in the absence of reducing agent L-cysteine is regarded 'free' activity 'Total' β-amylase activity is determined by extraction in reducing conditions, in the presence of L-cysteine.

RNA was extracted from grains following steeping and during micromalting, at anthesis and during development (weeks 1 to 5 post anthesis). RNA was blotted and probed for β-amylase mRNA, to see the stage during development and germination in which β-amylase is expressed. The northern blot (Figure 3.32) shows no β-amylase mRNA at anthesis, expression was observed from 1 week post anthesis to 5 weeks post anthesis with the majority of expression occurring at 3 and 4 weeks post anthesis. β-Amylase seems to have the potential to be expressed throughout germination (Figure 3.33).

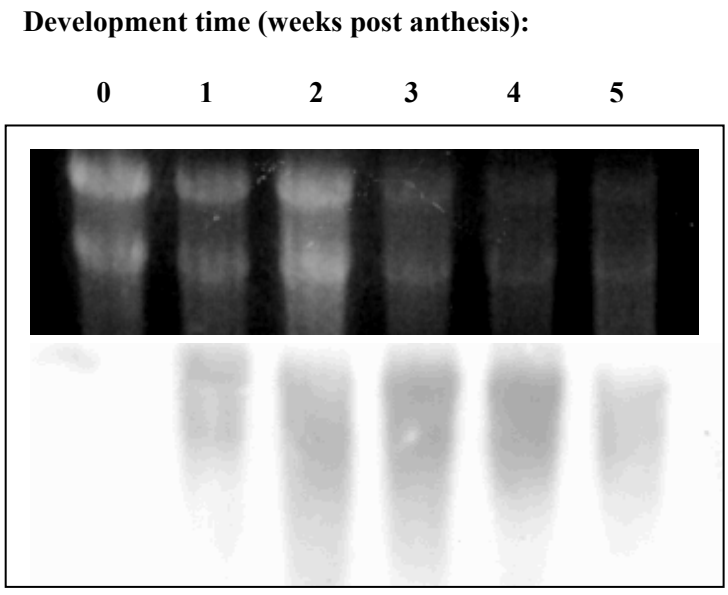


Figure 3.32: Northern blot analysis of β-amylase gene expression during grain development

Top: Ethidium bromide stained RNA

Bottom: Chemiluminescent detection of hybridised β-amylase RNA probe.

0 is at time of anthesis.

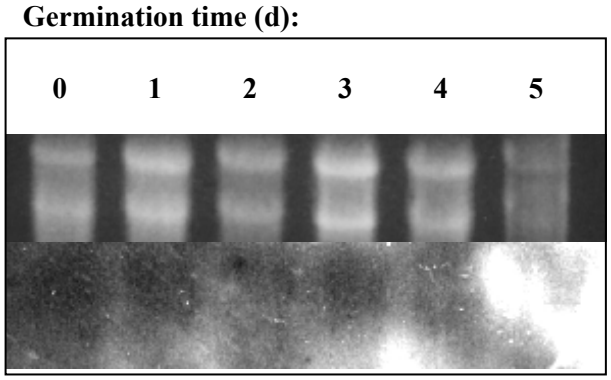


Figure 3.33: Northern blot analysis of β-amylase gene expression during grain germination

Top: Ethidium bromide stained RNA

Bottom: Chemiluminescent detection of hybridised β-amylase RNA probe.

0 is at time of maturation.

Malt flour had a 'free' β-amylase activity of ~ 700 mU g^{-1} following 48 h micromalting, this 'free' activity decreased to ~125 mU g^{-1} by 72 h, it stayed at this value for the following 48 h (Figure 3.34). The 'total' β-amylase activity increased from ~1000 mU g^{-1} at 48 h to ~ 1700 mU g^{-1} by 96 h micromalting. As the 'free' β-amylase activity levels were consistently low only 'total' activity was measured in single grains.

28 % of the individual 5 d aerobic commercial malt grains analysed had lower β-amylase activities than the lowest activity recorded for the 5 d aerobic micromalt (Figure 3.35). The 5 d aerobic micromalt also had ~ 20 % of grains with activities higher than the highest recorded for the commercial malt.

About 40 % grains from the 6 d aerobic micromalt had lower β-amylase activities than the lowest value obtained from the 5 d aerobic 1 d anaerobic micromalt (Figure 3.36). The 6 d aerobic micromalt also had ~ 20 % grains with higher β-amylase activity than the highest 5 d aerobic 1 d anaerobic data point, the highest β-amylase activity value of the 6 d aerobic micromalt was ~ 60 mU $grain^{-1}$ higher than the highest value of the 5 d aerobic 1 d anaerobic micromalt.

The statistical analysis presented in Table 3.10 shows the 5 d aerobic micromalt to have the highest mean and median as well as the lowest coefficient of variation. The mean β-amylase activity decreased after 5 d aerobic micromalting as the 6 d aerobic

micromalt and the 5 d aerobic 1 d anaerobic micromalt have mean values less than half of the mean calculated for the 5 d aerobic micromalt. The 6 d aerobic micromalt exhibited the largest standard deviation, range and coefficient of variation while the 5 d aerobic 1 d anaerobic malt had the lowest standard deviation.

The cumulative distribution curve (Figure 3.37) shows the lowest ($F(x) = 0$) and the highest ($F(x) = 1$) β-amylase activity values (mU grain^{-1}) to come from the 6 d aerobic malt. At $F(x) = 0$ the highest data point is obtained by the 5 d aerobic micromalt.

When β-amylase activity (mU g^{-1}) was calculated using the mean single grain β-amylase activity (mU grain^{-1}) and single grain weight (g) (Figure 3.38) the 5 d aerobic micromalt had a total β-amylase activity which was ~ 600 mU g^{-1} lower than was measure in the 120 h micromalt flour (Figure 3.34), other malt samples were lower in calculated activity.

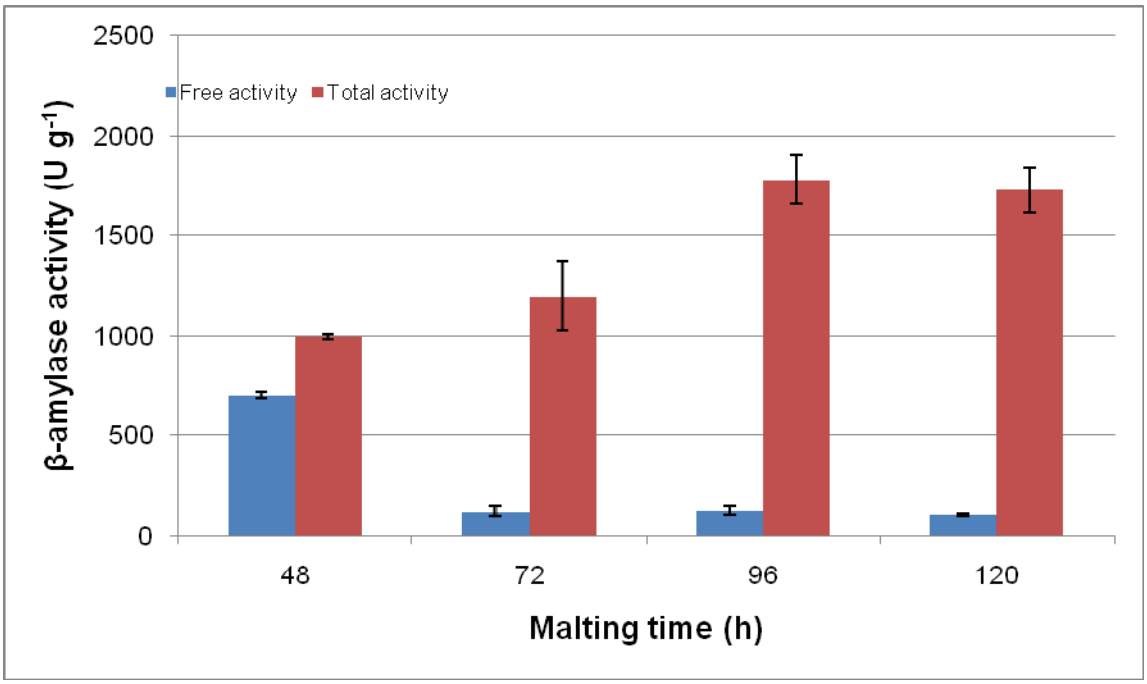


Figure 3.34: β-amylase activity (U g^{-1}) of kilned malt during 5 d aerobic germination in the micromalting unit, free activity represents extraction in the absence of L-cysteine and total activity represents extraction in the presence of L-cysteine, n = 3 tests on the same malt ± standard deviation

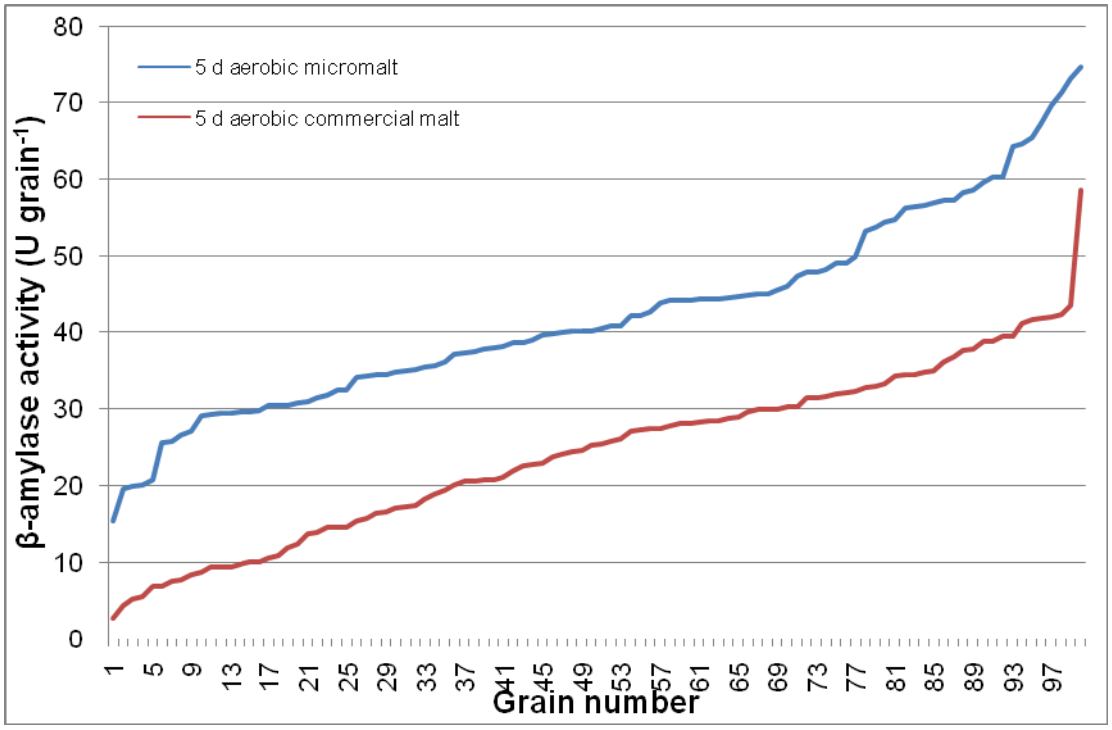


Figure 3.35: β-amylase activity (U grain^{-1}) of 100 single kilned malt grains produced following 5 d aerobic germination either in the micromalting unit at Heriot-Watt University or commercially produced at Simpsons Maltings

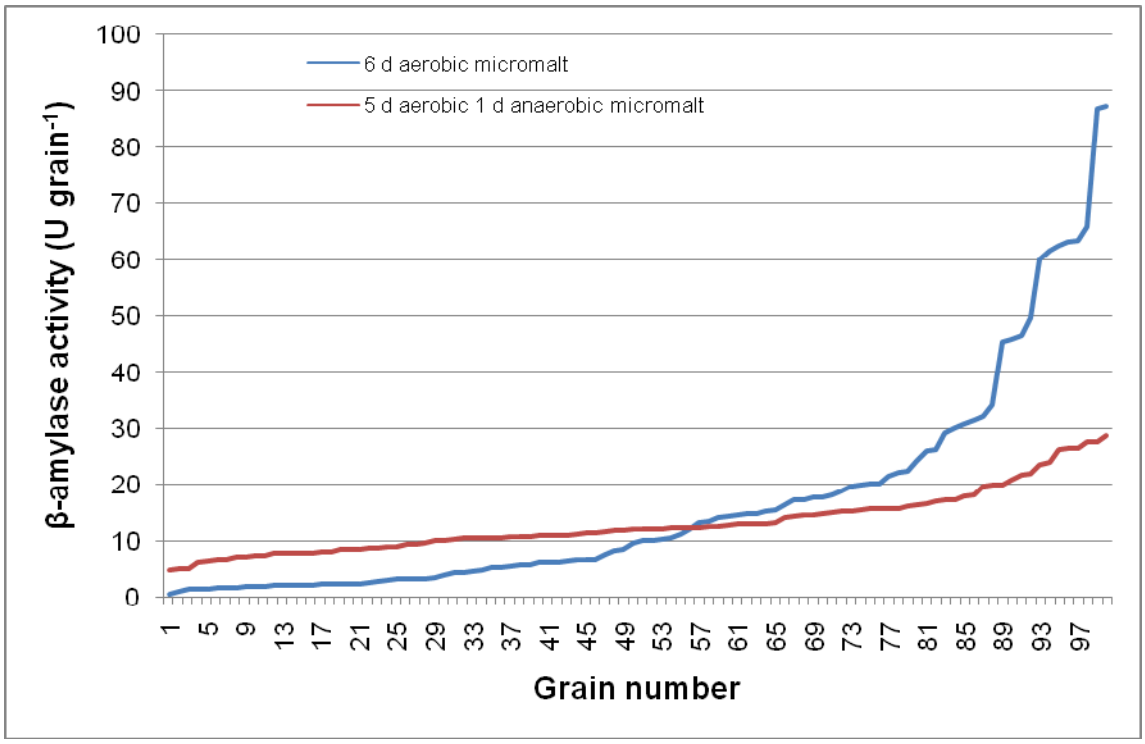


Figure 3.36: β-amylase activity (U grain^{-1}) of 100 single kilned malt grains produced following 6 d aerobic germination in the micromalting unit at Herriot-Watt University or 5 d aerobic micromalting and 1 d anaerobic germination

Statistic	5 d aerobic micromalt	5 d aerobic commercial malt	6 d aerobic micromalt	5 d aerobic 1 d anaerobic micromalt
Mean	42.35	24.24	16.60	13.05
Standard deviation	12.70	11.18	19.25	5.46
Median	40.33	25.43	9.89	12.01
Range	59.06	55.85	86.67	23.88
%cv	30.00	46.11	115.94	41.84

Table 3.10: Statistical analysis for single grain β-amylase activity (U grain^{-1}) of 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt. All micromalt was produced at the micromaltings at Heriot-Watt University, commercial malt was produced at Simpson's maltings, Berwick-Upon-Tweed

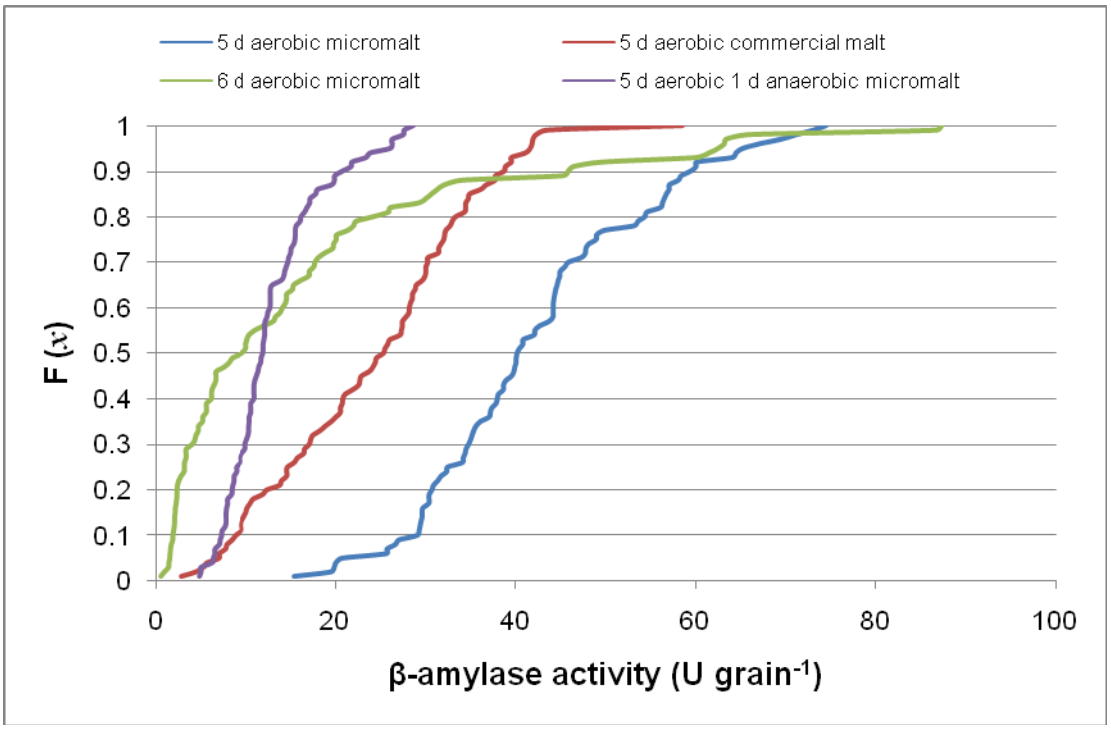


Figure 3.37: Cumulative distribution function (CDF) of β-amylase (U grain^{-1}) for 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt

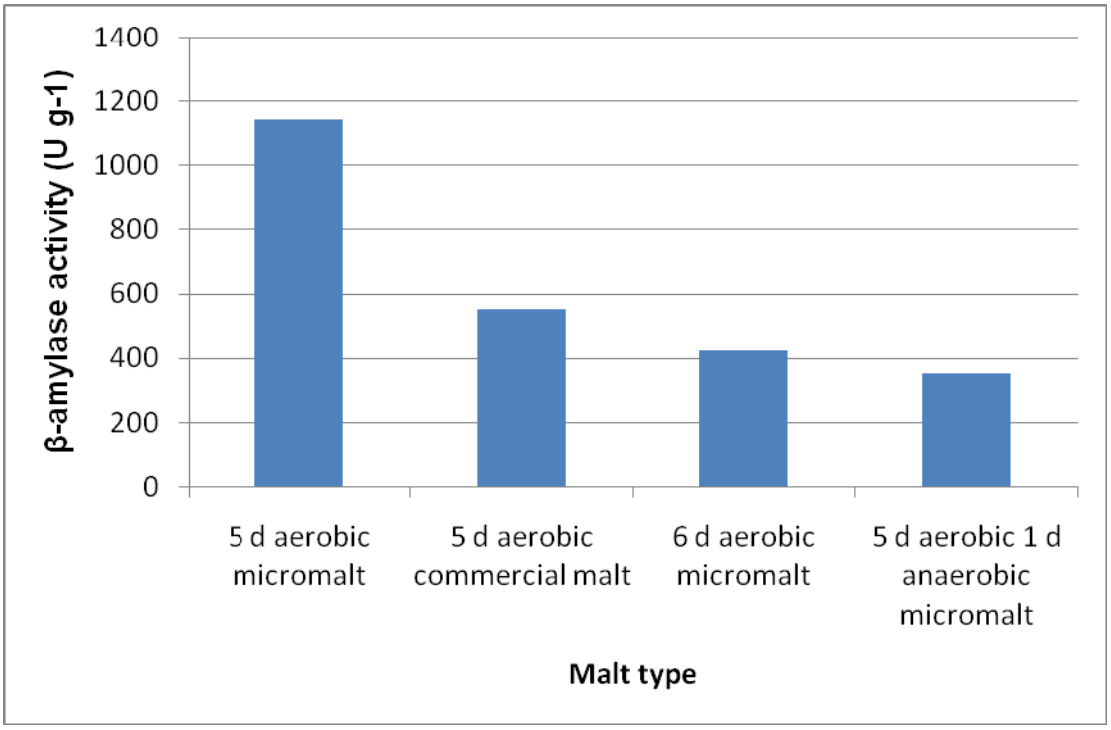


Figure 3.38: β-amylase activity (U g^{-1}) of 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic malt and 5 d anaerobic 1 d anaerobic micromalt when calculated using mean β-amylase activity (U $grain^{-1}$) values (Table 3.10) and mean single grain weight (g) values (Table 3.2)

3.4.3. Limit dextrinase

Limit dextrinase is important in the brewing and distilling industry as it has the potential to convert non fermentable dextrins containing α-1, 6 bonds into fermentable sugars.

RNA was extracted from grains following steeping and during germination in a micromalting unit. RNA was blotted and probed for limit dextrinase mRNA, thus establishing the time of limit dextrinase expression. The northern blot (Figure 3.39) shows limit dextrinase mRNA to be present following steeping and throughout germination. It is apparent that limit dextrinase mRNA is expressed mainly at 2 d aerobic germination during micromalting.

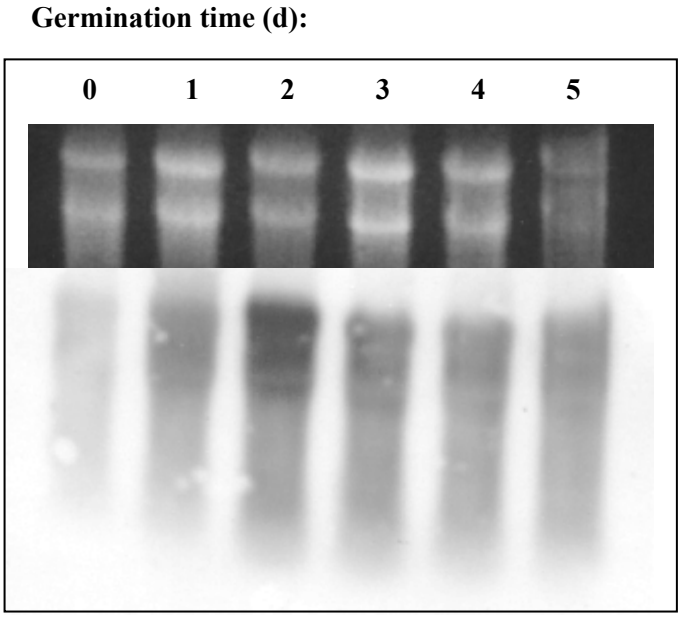


Figure 3.39: Northern blot analysis of limit dextrinase gene expression during grain germination

Top: Ethidium bromide stained RNA

Bottom: Chemiluminescent detection of hybridised α-amylase RNA probe.

0 is mature grains at the start of the germination process.

Both 'free' and 'total' limit dextrinase activity of malt flour was found to increase between 48 and 120 h of micromalting (Figure 3.40). The 'free' limit dextrinase activity increased from ~ 60 mU g^{-1} at 48 h to ~ 170 mU g^{-1} by 120 h micromalting. The 'total' limit dextrinase activity increased from ~ 125 mU g^{-1} at 48 h micromalting to ~ 365 mU g^{-1} at 120 h. Following 120 h micromalting only ~ 47 % of 'total' limit dextrinase activity was 'free'.

When single grain limit dextrinase activity was measured ~ 64 % of single grains from the 5 d aerobic micromalt had 'free' activity lower than 1.5 mU $grain^{-1}$, the lowest value of the 5 d aerobic commercial malt (Figure 3.41). 10 grains from the 5 d aerobic micromalt had 'free' activities higher than 7.1 mU $grain^{-1}$, the highest value measured for the 5 d aerobic commercial malt. Despite having similar highest and lowest 'total' activity values (mU $grain^{-1}$), a larger proportion of the 5 d aerobic micromalt grains had higher activities than the 5 d aerobic commercial malt grains (Figure 3.42).

A larger proportion of 5 d aerobic 1 d anaerobic malt grains had higher 'free' limit dextrinase activity than the 6 d aerobic micromalt grains (Figure 3.43). The lowest

single grain ‘total’ limit dextrinase activity of 5 d aerobic 1 d anaerobic micromalt was 5 mU grain^{-1} higher than the lowest activity value obtained for the 6 d aerobic micromalt (Figure 3.44). ~32 % of the 6 d aerobic micromalt grains measured had activities lower than the lowest activity value of the 5 d aerobic 1 d anaerobic micromalt.

Table 3.11 shows the 5 d aerobic 1 d anaerobic malt had the highest mean ‘free’ limit dextrinase activity while the 5 d aerobic micromalt had the lowest. The highest standard deviation and range of ‘free’ limit dextrinase activity occurred when the 6 d aerobic micromalt was analysed. The highest coefficient of variation occurred in 5 d aerobic micromalt grains.

The highest mean 'total’ limit dextrinase activity (mU grain^{-1}) was also observed in 5 d aerobic 1 d anaerobic malt grains (Table 3.12). The highest range and standard deviation was found for 6 d aerobic micromalt grains. The 5 d aerobic commercial malt exhibited the highest coefficient of variation.

Cumulative distribution patterns (Figures 3.45 and 3.46) show the highest distribution of ‘free’ and ‘total’ limit dextrinase activity to be in the 5 d aerobic 1 d anaerobic micromalt grains.

When the ‘free’ and ‘total’ limit dextrinase activity (mU g^{-1}) was calculated using the mean single grain limit dextrinase activities (mU grain^{-1}, Tables 3.11 and 3.12) and the mean single grain weight (Table 3.2) the 5 d aerobic micromalt had ‘free’ and ‘total’ activities which were each ~ 100 mU g^{-1} lower (Figure 3.47) than was measured in the malt flour (Figure 3.40). Interestingly the 5 d aerobic 1 d anaerobic micromalt had a calculated ‘free’ limit dextrinase activity ~ 100 mU g^{-1} higher than what was measured in the 5 d aerobic micromalt flour.

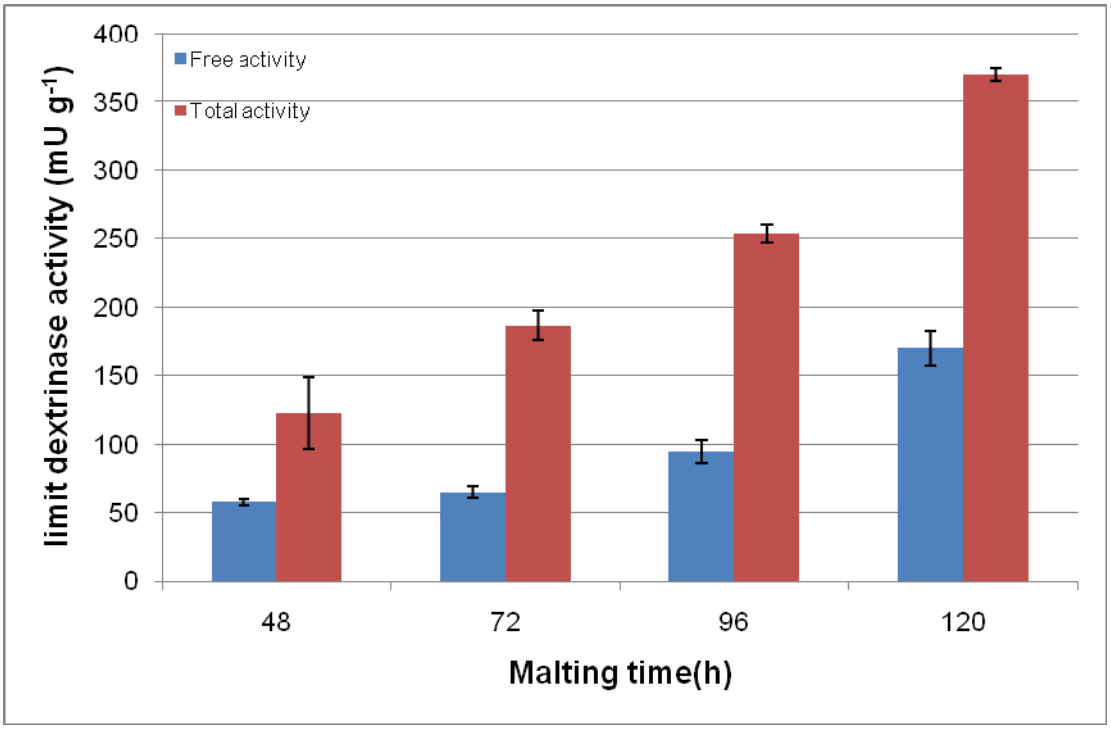


Figure 3.40: Limit dextrinase activity (mU g^{-1}) of kilned malt during 5 d aerobic germination in the micromalting unit, free activity represents extraction in the absence of DTT, total activity represents extraction in the presence of DTT, n = 3 tests on the same malt ± standard deviation

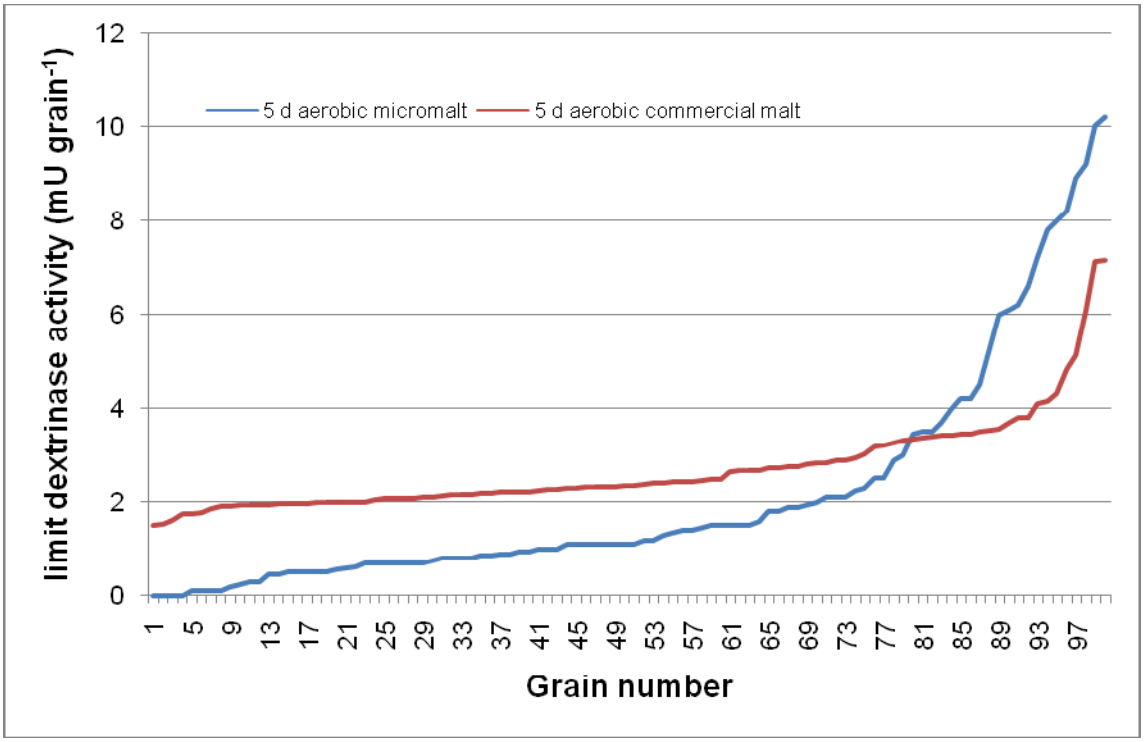


Figure 3.41: 'Free' limit dextrinase activity (mU $grain^{-1}$) of 100 single kilned malt grains produced following 5 d aerobic germination either in the micromalting unit at Herriot-Watt University or commercially produced at Simpsons Maltings

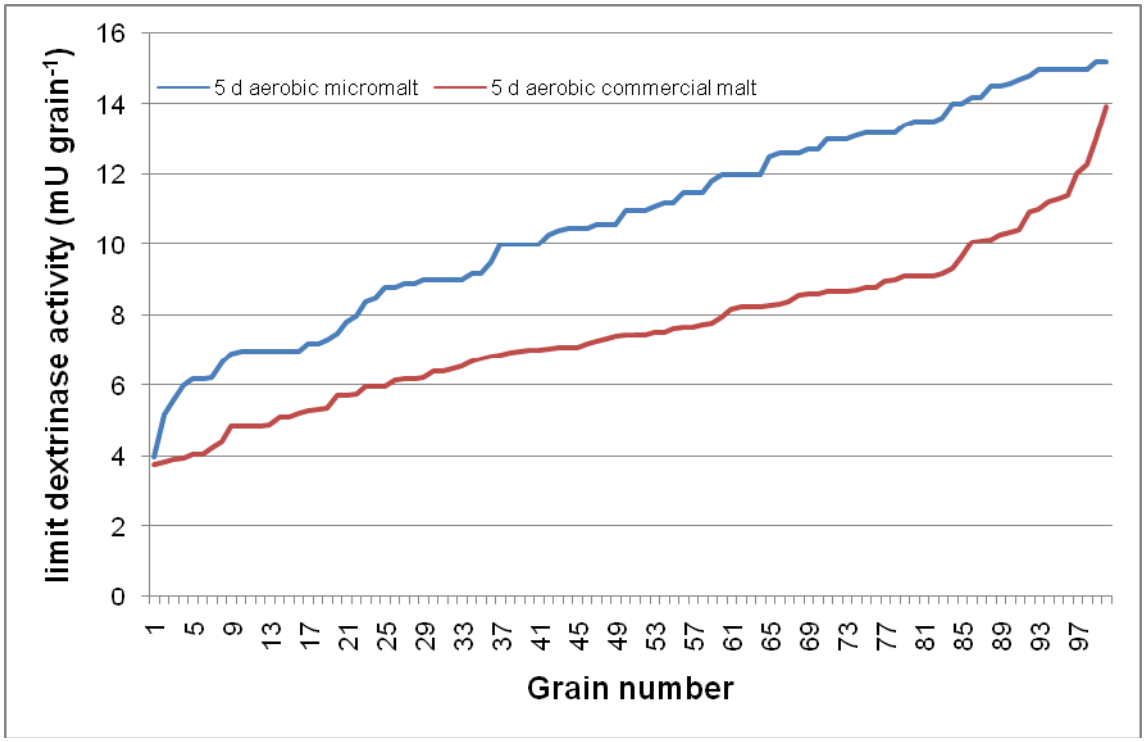


Figure 3.42: 'Total' limit dextrinase activity (mU grain^{-1}) of 100 single kilned malt grains produced following 5 d aerobic germination either in the micromalting unit at Herriot-Watt University or commercially produced at Simpsons Maltings

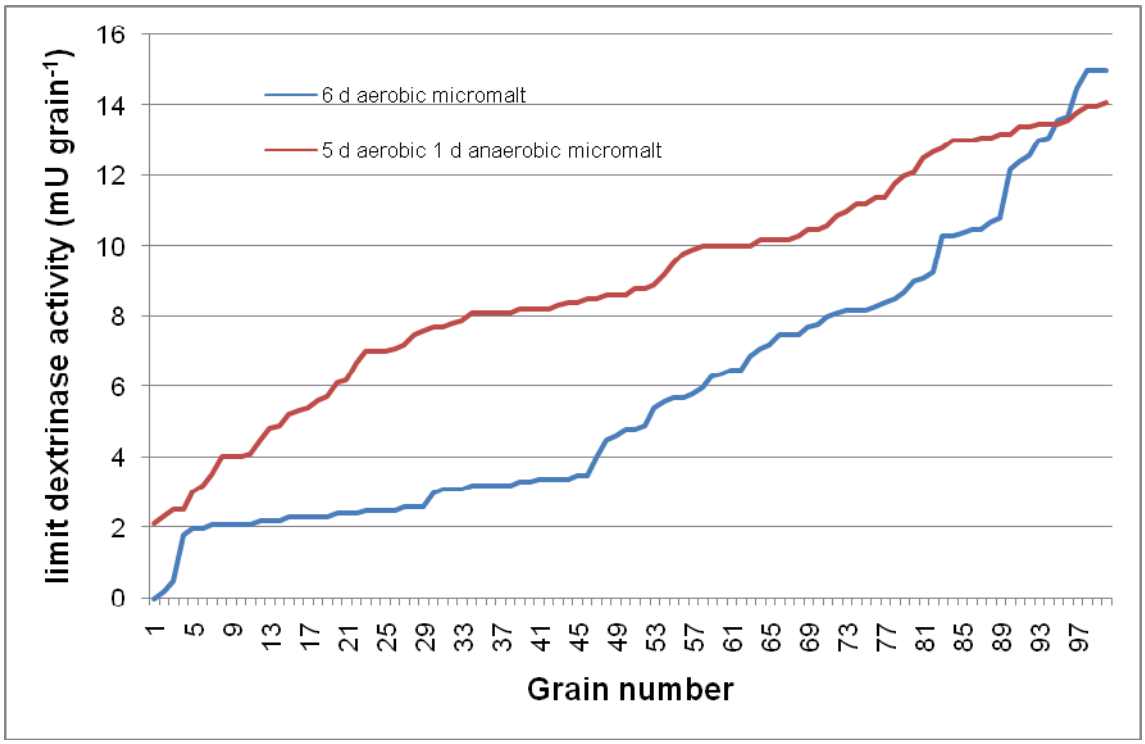


Figure 3.43: 'Free' limit dextrinase activity (mU grain^{-1}) of 100 single kilned malt grains produced following 6 d aerobic germination in the micromalting unit at Herriot-Watt University or 5 d aerobic micromalting and 1 d anaerobic germination

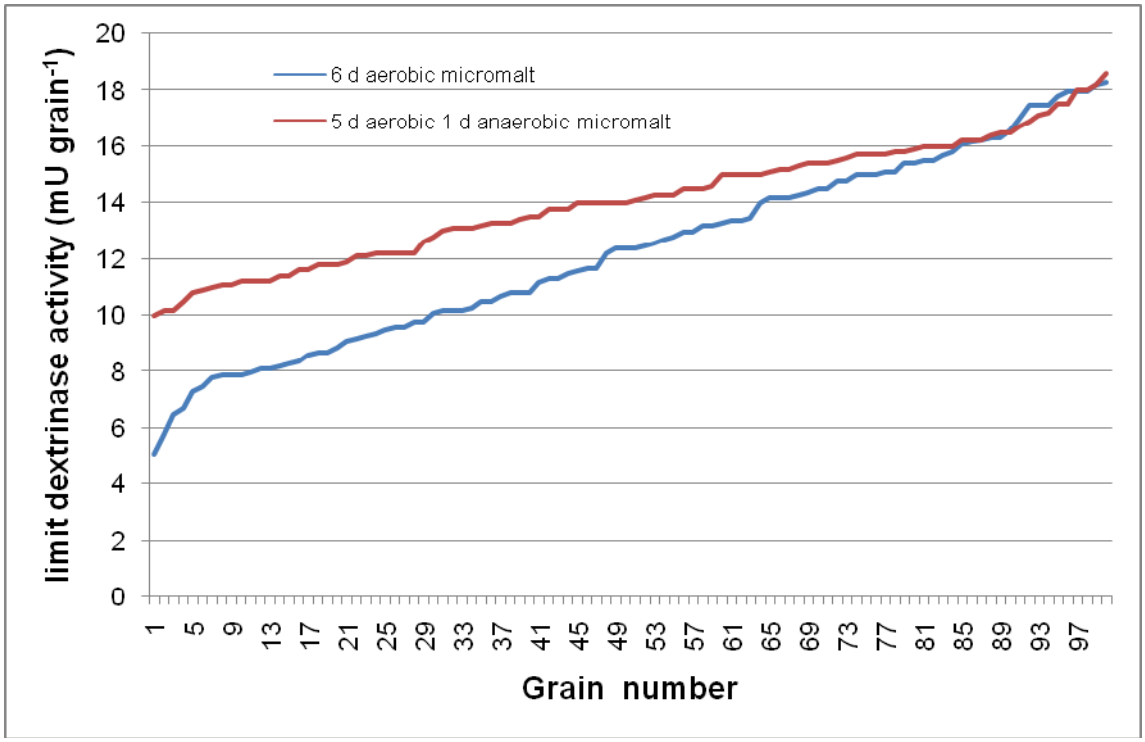


Figure 3.44: 'Total' limit dextrinase activity (mU grain^{-1}) of 100 single kilned malt grains produced following 6 d aerobic germination in the micromalting unit at Herriot-Watt University or 5 d aerobic micromalting and 1 d anaerobic germination

Statistic	5 d aerobic micromalt	5 d aerobic commercial malt	6 d aerobic micromalt	5 d aerobic 1 d anaerobic micromalt
Mean	2.15	2.70	5.88	8.95
Standard deviation	2.43	1.02	3.88	3.19
Median	1.10	2.33	4.80	8.70
Range	10.20	5.66	15.00	12.00
%cv	112.90	37.78	65.98	35.64

Table 3.11: Statistical analysis for single grain 'free' limit dextrinase activity (mU grain^{-1}) of 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt. All micromalt was produced at the micromaltings at Heriot-Watt University, commercial malt was produced at Simpson's maltings, Berwick-Upon-Tweed

Statistic	5 d aerobic micromalt	5 d aerobic commercial malt	6 d aerobic micromalt	5 d aerobic 1 d anaerobic micromalt
Mean	10.77	7.59	12.29	14.06
Standard deviation	2.87	2.16	3.34	2.10
Median	11.00	7.45	12.40	14.05
Range	11.20	10.15	13.20	8.60
% cv	26.62	28.52	27.18	14.91

Table 3.12: Statistical analysis for single grain 'total' limit dextrinase activity (mU grain^{-1}) of 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt. All micromalt was produced at the micromaltings at Heriot-Watt University, commercial malt was produced at Simpson's maltings, Berwick-Upon-Tweed

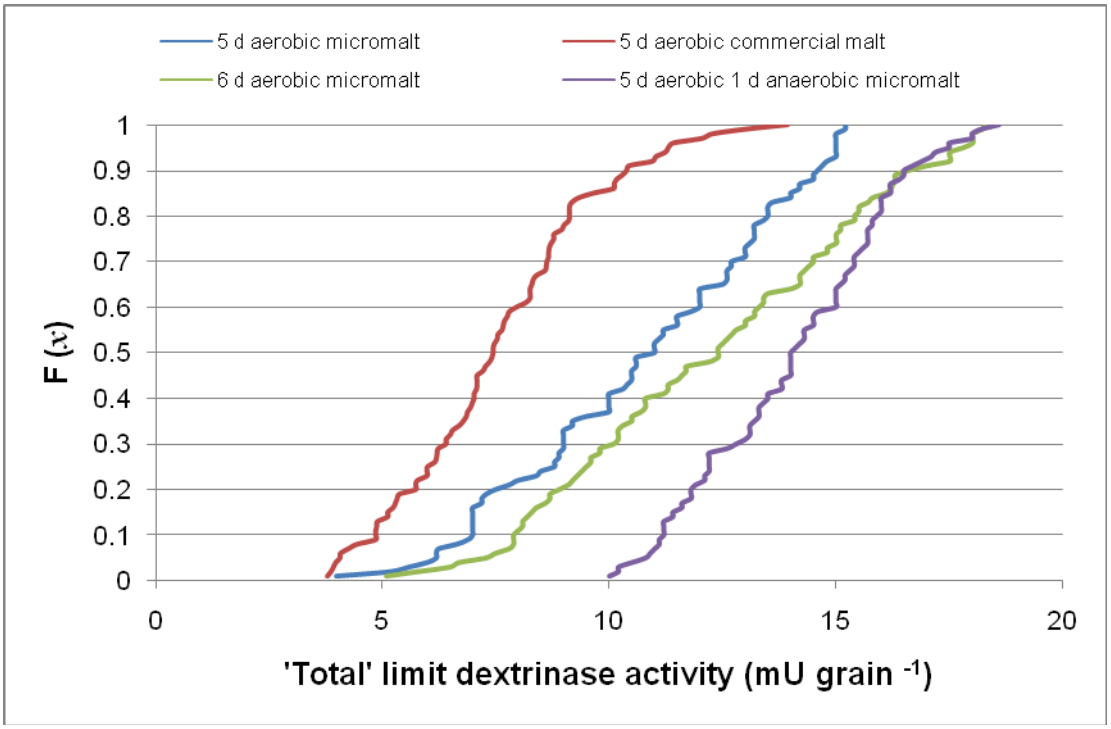


Figure 3.45: Cumulative distribution function (CDF) of 'total' limit dextrinase activity (mU grain^{-1}) for 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt

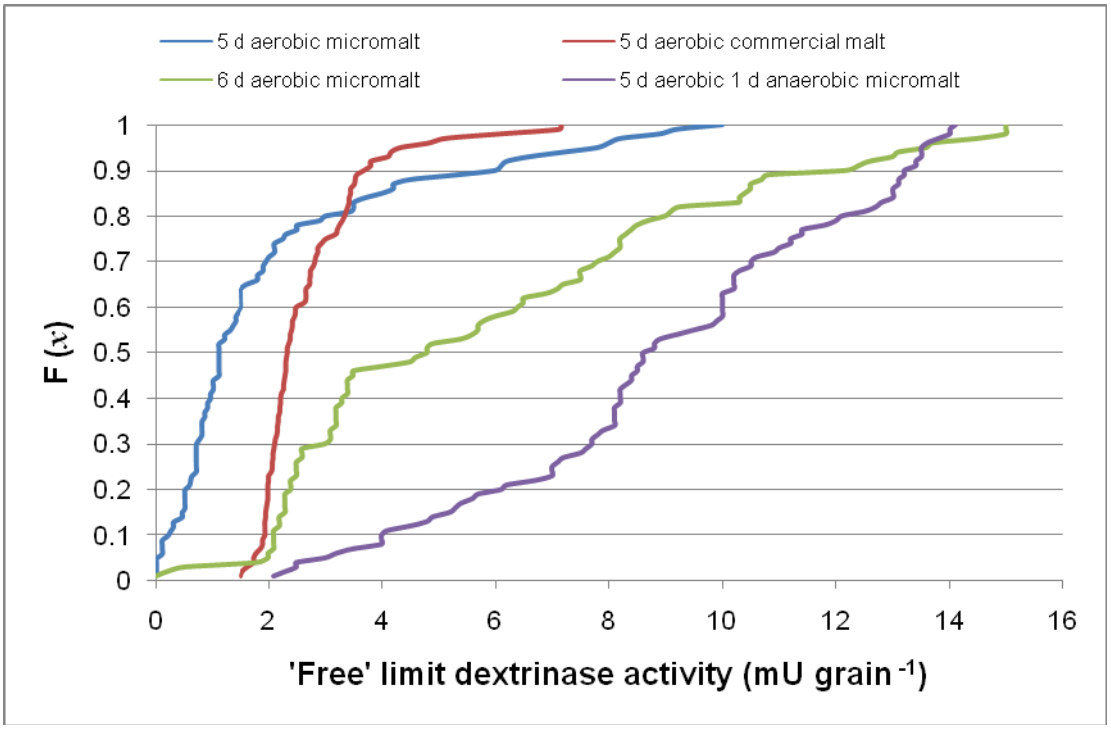


Figure 3.46: Cumulative distribution function (CDF) of 'free' limit dextrinase activity (mU grain^{-1}) for 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt

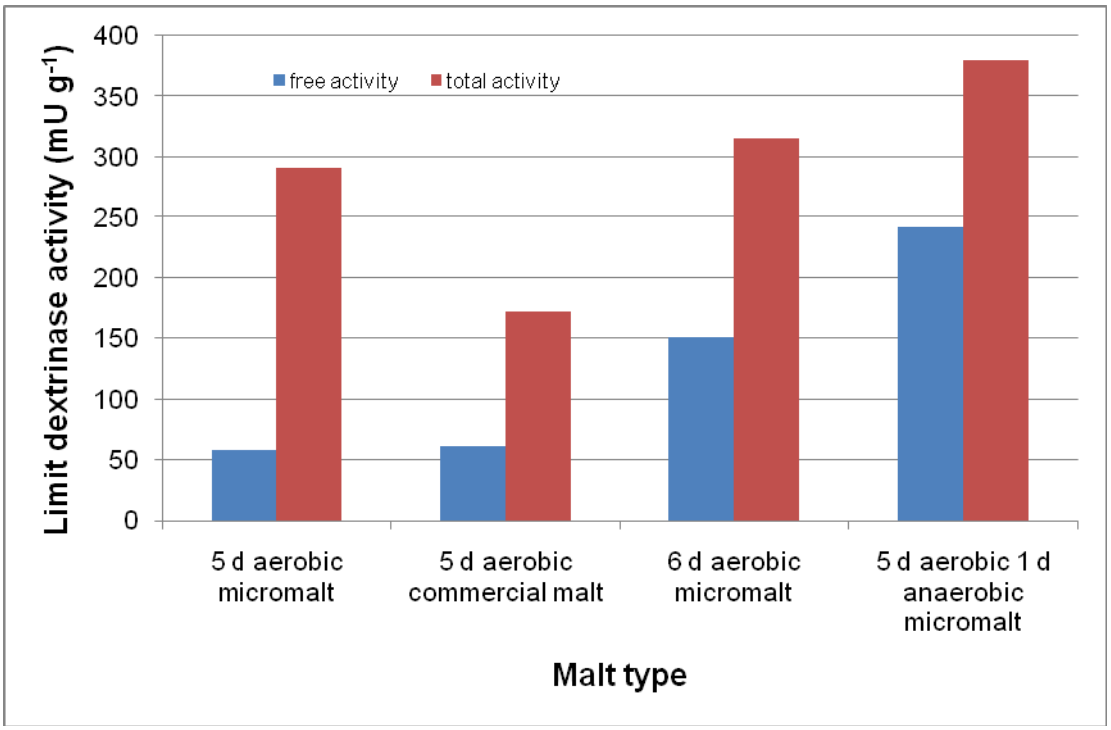


Figure 3.47: 'Free' and 'total' limit dextrinase activity (mU g^{-1}) of 5 d aerobic micromalt, 5 d aerobic commercial malt, 6 d aerobic malt and 5 d anaerobic 1 d anaerobic micromalt when calculated using mean 'free' limit dextrinase activity (mU grain^{-1}) values (table 3.11), mean 'total' limit dextrinase (mU grain^{-1}) values (Table 3.12) and mean single grain weight (g) values (Table 3.2)

3.4.4. Limit dextrinase inhibitor

In order to assay for limit dextrinase inhibitor activity, a source of limit dextrinase was required. Hence limit dextrinase was partially purified from malted grain. *Optic* barley was micromalted for 8 d aerobically; the protein was extracted and dialysed (see section 2.8.1). Proteins present in the crude extract were separated using DEAE Sepharose Cation Exchange chromatography. The presence of limit dextrinase in the fractions collected during chromatography was determined by measuring the absorbance at 510 nm of supernatant following assay with red pullulan, figure 3.48 shows the limit dextrinase activity to be present in fractions 37 to 41. These fractions were pooled, Figure 3.49 is an SDS-PAGE showing the protein content of crude extract and partially purified limit dextrinase, limit dextrinase has a molecular weight of 100 kDa.

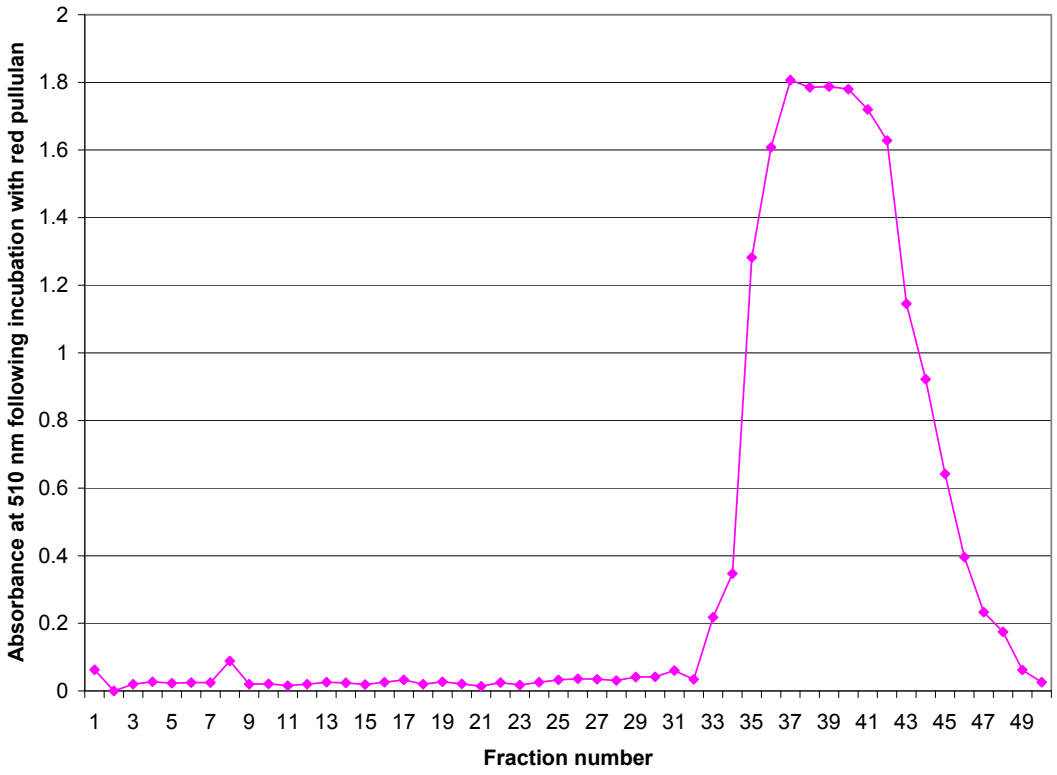


Figure 3.48: Partial purification of limit dextrinase by DEAE Sepharose Cation Exchange chromatography. Limit dextrinase activity of eluted fractions is shown

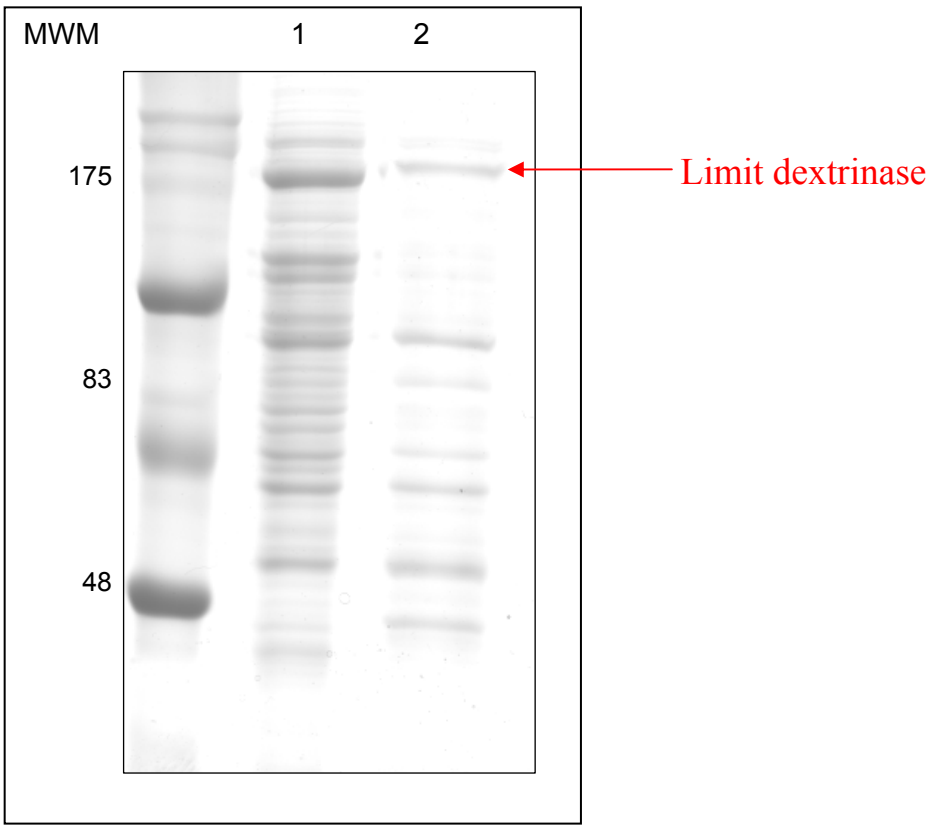


Figure 3.49: SDS-PAGE, MW represents molecular weight markers in kDa.
Lane 1: Crude extract of 8 d micromalted barley.
Lane 2: Partially purified limit dextrinase.
Arrow points to band corresponding to known size of limit dextrinase protein.

Limit dextrizyme assays were used to measure the % inhibition of mature grain crude extract had on partially purified limit dextrinase. Crude extract was the supernatant prepared from single grains by suspending mature grain flour (ground in a pestle and mortar) in 500 µl 0.02 M sodium acetate buffer pH 5.2 for 10 m while shaking and centrifuging at 10 000 x g for 10 m. 50 µl crude extract was added to 50 µl partially purified limit dextrinase in 400 µl 100 mM sodium maleate, 0.02 % sodium azide (Megazyme specified extraction buffer) and allowed to incubate at room temperature for 30 m. Limit dextrizyme assays were then carried out as described in section 2.5.6.

50 µl mature grain crude extract had the ability to inhibit between 40 and 100 % of partially purified limit dextrinase activity (5 mU per 50 µl) (Figure 3.50), on average 63.77 % ± 12.64 of 50 µl partially purified limit dextrinase was inhibited by 50 µl crude extract (Table 3.13).

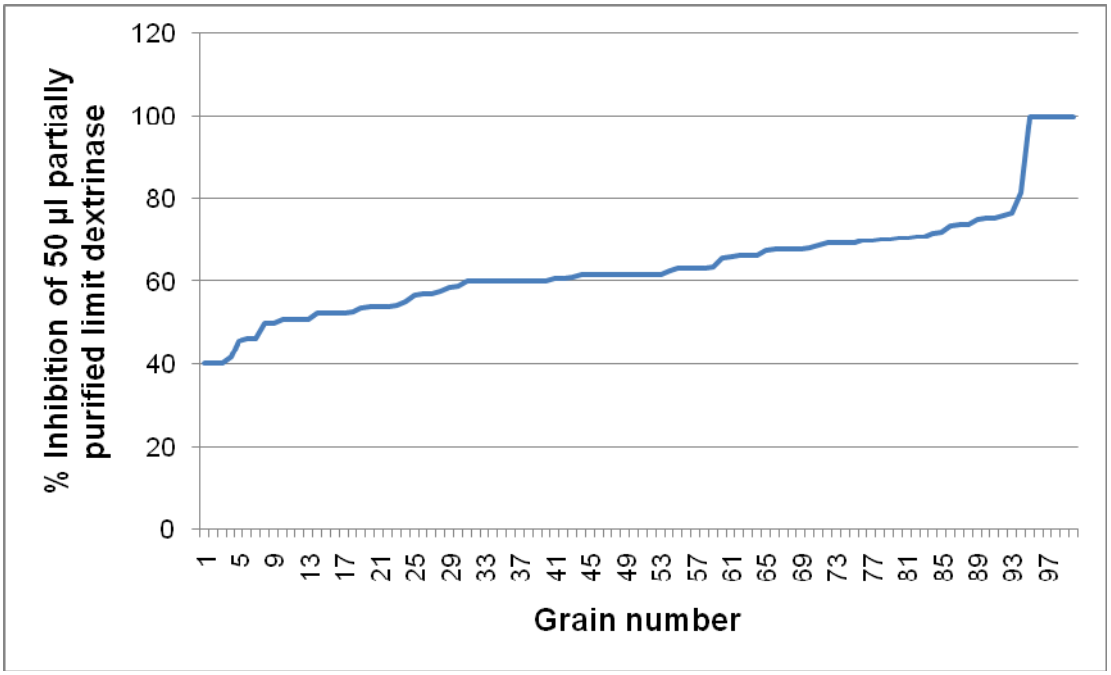


Figure 3.50: Percent inhibition of 50 µl partially purified limit dextrinase activity (100 % limit dextrinase activity = 5 mU per 50 µl) by 50 µl crude extracts prepared from 100 single mature *Optic* barley grains

Statistic	Mature grain crude extract
Mean	63.77
Standard deviation	12.64
Median	61.54
Range	60
% cv	19.81

Table 3.13: Statistical analysis for % inhibition of 50 µl partially purified limit dextrinase - activity (100 % limit dextrinase activity = 5 mU per 50 µl) by 50 µl crude extracts prepared from 100 single mature *Optic* barley grains

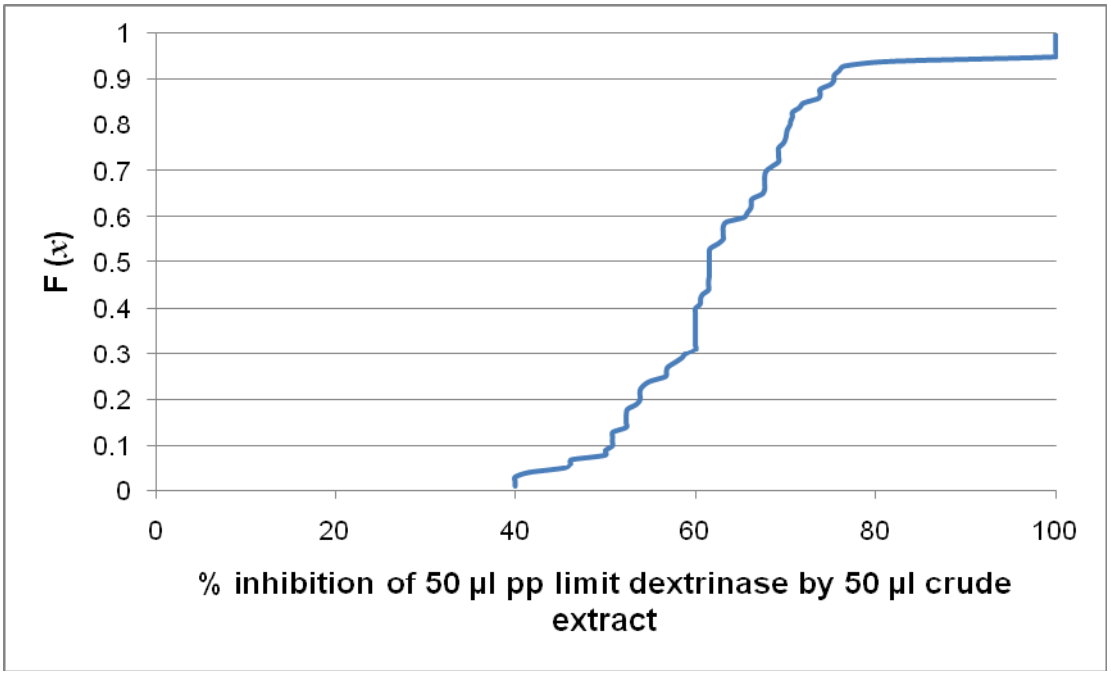


Figure 3.51: Cumulative distribution function (CDF) of % inhibition of 50 µl partially purified limit dextrinase activity (100 % limit dextrinase activity = 5 mU per 50 µl) by 50 µl crude extracts prepared from 100 single mature *Optic* barley grains

Attempts were made to purify limit dextrinase inhibitor from crude extract. The extract was initially heated to denature many unwanted proteins, it was then passed through a CM Sepharose column, and fractions containing activity were pooled and run through either a Mono-Q column or a gel filtration column as shown in Table 3.14. Despite changes in the NaCl gradient, the loading time and yields of limit dextrinase inhibitor were consistently low (when expressed as recovery %).

Purification stage	Total volume (ml)	Total protein (mg)	Total inhibitory activity (antiunits)	Specific activity (antiunits/ µg)	Recovery (%)
Eluted by salt gradient 0 to 500 mM NaCl over 1 h					
Crude extract	750	5690	7580779	1.33	100
Heated extract	700	4550	6519470	1.43	86
After CM Sepharose	20	191	8220	0.043	0.126
After MonoQ	6	0.87	373	0.43	0.045
Eluted by salt gradient 0 to 300 mM NaCl over 1 h					
Crude extract	750	3000	400000	0.133	100
Heated extract	700	1270	250000	0.197	62.5
After CM Sepharose	32	12.35	4642	0.375	1.86
After gel filtration	3	0.006	6.16	1.03	0.133
Sample loaded slowly onto column over 16 h at speed 26 ml h^{-1}, eluted by salt gradient 0 to 500 mM NaCl over 8 h					
Crude extract	700	770	229667	0.298	100
Heated extract	650	520	216666	0.417	94
After CM Sepharose	50	5.48	12500	2.28	5.77

Table 3.14: Strategy used in the partial purification of limit dextrinase inhibitor protein and the resulting total volume, total protein, total inhibitory activity, specific activity and recovery at each stage

In order to investigate the problem causing the low recovery of LDI, the crude extract was run through a gel filtration column. Prior to the analysis of the crude extract standards of known molecular weight (blue dextran: 2000 kDa, BSA: 66.3 kDa and Cytochrome c: 12.4 kDa) were used to calibrate the column. Individual fractions were run on an SDS-PAGE (Figure 3.44 B), a western blot was also performed on fractions, and probed using the high pI limit dextrinase inhibitor antibody (Figure 3.44 C). Limit dextrinase inhibitor was found in fractions 2 to 5.

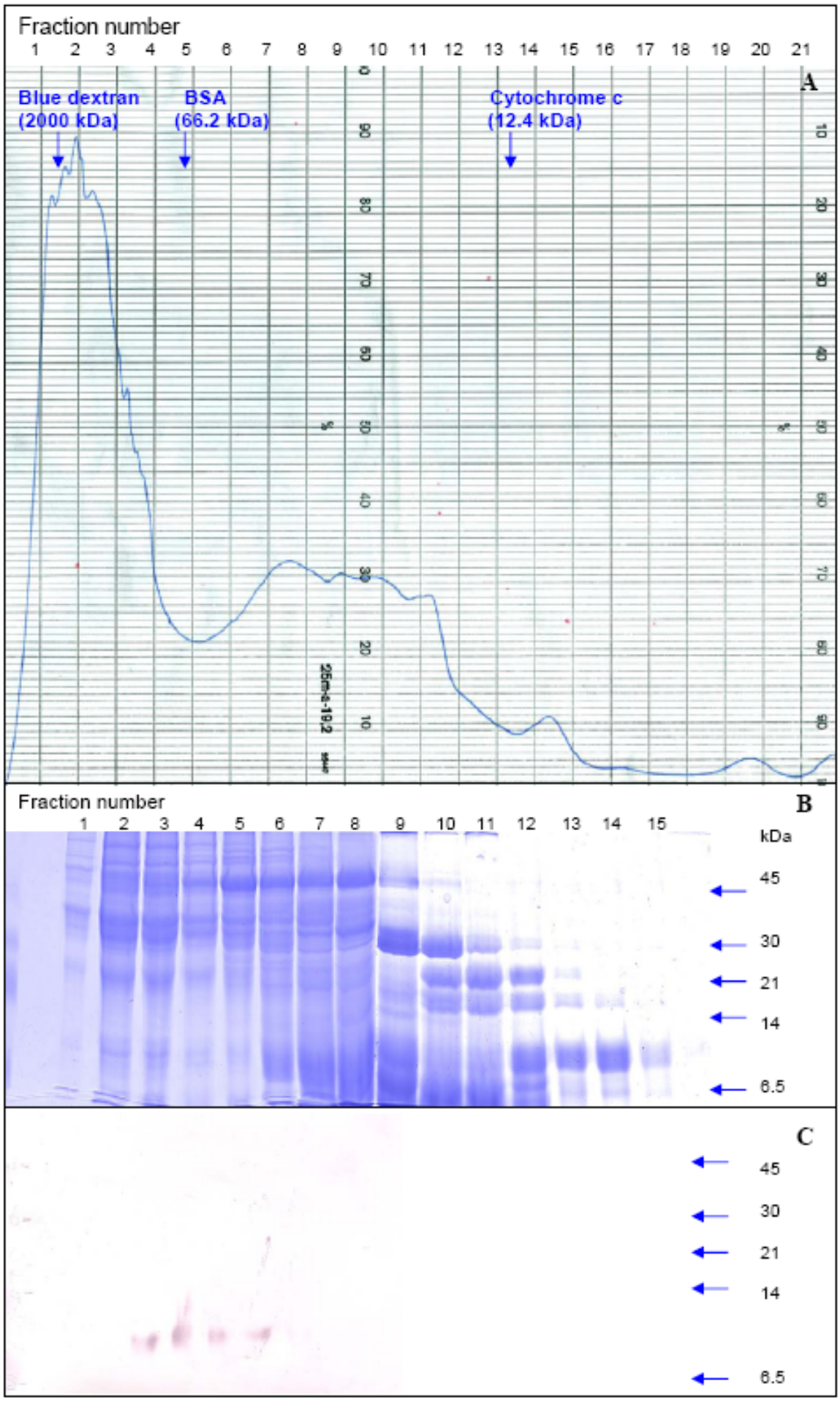

Fraction number
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
A
Blue dextran
(2000 kDa)
BSA
(66.2 kDa)
Cytochrome c
(12.4 kDa)
%
Fraction number
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
kDa
45
30
21
14
6.5
C
45
30
21
14
6.5

Figure 3.52: Fractionation of limit dextrinase inhibitor using FPLC liquid chromatography gel filtration with Superose-12 (Previous page)

A: OD 280 of elute from FPLC, OD 1.0, flow rate 0.5 ml min-1, each line on the horizontal axis represents 1 ml.

B: SDS-PAGE of 1 ml fractions 1 to 15, MWM represents the molecular weight marker.

C: Western blot prepared from the SDS-PAGE (containing 1 ml fractions 1 to 15) probed specifically with limit dextrinase inhibitor antibody.

50 µl fractions from the FPLC were incubated with 50 µl partially purified limit dextrinase (activity = 6.3 mU per 50 µl) and the % inhibition calculated (Figure 3.54). The highest amount of inhibition occurred in fractions 1 to 4.

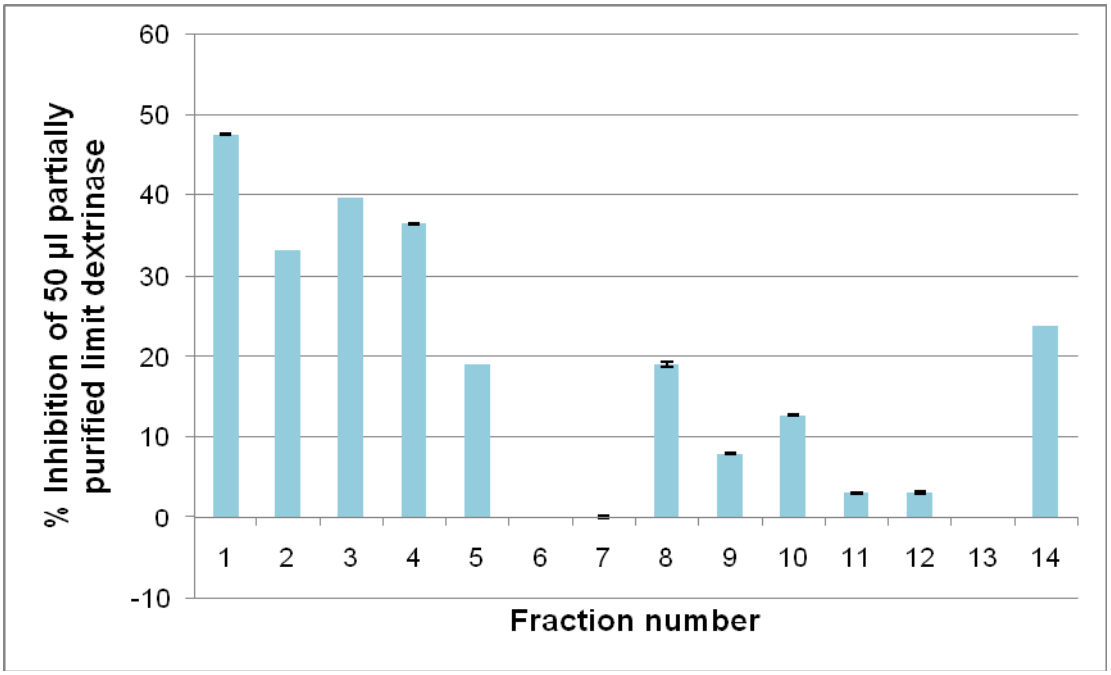


Figure 3.53: % inhibition of 50 µl partially purified limit dextrinase (100 % activity = 6.3 mU) by 50 µl of gel filtration fractions, n = 3 ± standard deviation

Chapter 4. Discussion

4.1. Homogeneity

Traditional analysis of malt, for example as recommended by the IoB, gives no indication of the distribution of starch breakdown into fermentable sugars or indeed the heterogeneity of enzyme activities between individual malt grains, which are responsible for this modification.

During standard method analysis an amount (10 to 50 g) of each sample is milled and extracted turning a heterogeneous sample into a homogeneous mixture thus concealing the original heterogeneity of modification in the grain population of the malt sample.

Traditional analysis does not expose the varied distribution of parameters such as nitrogen concentration, fermentable sugar content and enzyme activity, lack of knowledge of such variable distributions can lead to brewhouse problems when a malt which is expected to process satisfactorily fails to do so. For example, theoretically a malt sample with an average limit dextrinase activity of 30 mU g^{-1} can have 20 mU g^{-1} in 80 % of its grains and 70 mU g^{-1} in the remaining 20 % of grains, such a malt may produce a wort with a lower yield of fermentable sugars than anticipated.

In order to investigate the heterogeneity of enzyme activity, as well as the content of nitrogen and fermentable sugars of malt samples, existing methods were adapted to measure single grains. Using these adapted methods it was then possible to investigate the differences in individual grain heterogeneity between 5 d aerobic malt as prepared in the micromalting unit at Heriot-Watt University and commercially produced 5 d aerobic malt made at Simpsons Maltings in Berwick-Upon-Tweed. It was also possible to observe the physiological response of 24 h anaerobiosis on 5 d aerobically germinated micromalt because some grains in commercial malting beds may be anaerobic.

For each parameter measured in this investigation 100 single grains of each malt type were analysed, excluding the fermentable sugar content analysis, as HPLC is a complex and time consuming procedure only 20 grains of each malt type were

analysed. To achieve a more accurate understanding of the degree of inhomogeneity of samples larger population sizes could have been studied.

4.2. Grain weight

As expected, individual grains in a population of 100 weighed for each malt type were subject to considerable variability (section 3.1.2).

The endosperm will have a major influence on the grain weight as it constitutes a high proportion of the barley grain. As a result genes which effect endosperm growth during development and maturation will inevitably have an effect on the grain size. The embryo acts as a sink for nutrients supplied by the barley plant during early development, endosperm and testa growth in early development have a primary role in determining the size of the postfertillisation embryo sac, which in turn influences embryo and grain size (Scott and Close, 1997).

Variations in the seed mass of faba bean, pea and cereals have been strictly correlated with cell number differences, not cell size. Mechanisms which dictate grain size and weight must control the extent of cell proliferation in the embryo and endosperm during development (Wobast *et al*., 1999).

The germination time of barley has been related to grain size. Grain size can increase the germination time by ~ 17 min mg^{-1} (Ellis *et al*., 1998). The positioning of a barley grain on the ear has also been shown to alter germination time in the order top > bottom > middle, with grains at the top of the ear having the slowest germination rate (Yin *et al*., 2001). These findings support suggestions that the positioning of a grain on an ear will have an effect on the weight of the grain.

When looking at values calculated using single grain weight (section 3.1.2), it is important to consider that the lower the mean grain weight the higher the number of grains would be used per g malt flour. So even though the 5 d aerobic commercial malt has the highest mean grain weight (Table 3.2), the 5 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt will have a higher number of grains per g flour and

thus grain to grain variation would have a bigger impact on these malts. In this thesis the mean individual grain value for each parameter has been used to calculate the value expected per g of malt flour or litre of wort using mean individual grain weight.

4.3. pH of wort

The pH of wort prepared using the 5 d aerobic micromalt was similar to the pH of the wort prepared using the 5 d aerobic commercial malt despite the processing differences (Table 3.4). Wort prepared from 6 d aerobic micromalt was 0.2 pH units higher than worts made following 5 d aerobic malting. The pHs of worts made from aerobic malts were commercially acceptable as specified by the IOB. The wort made from 5 d aerobic 1 d anaerobic micromalt had a pH value of 4.69, a value almost 1 pH unit lower than worts made from 5 d aerobic malts.

pH regulation is the result of a complex interaction of ion transport, H^+ buffering, H^+ consumption and H^+ producing reactions. Anoxic cells experience an energy crisis, as an early response the pH of the cytoplasm is reduced by roughly 0.5 pH unit (Felle. 2005). There are several plausible explanations for this cytoplasmic decrease:

1. Pump deactivation, accompanied by H^+ leakage into the cytoplasm from outside the vacuole.
2. Anion channel activation with simultaneous cytoplasmic acidification and membrane depolarisation.
3. Lactic acid production.
4. A switch in metabolism requiring a new pH set point, it is postulated that as anaerobic machinery produces insufficient amounts of ATP, a new pH is set to ensure the proper functioning of enzymes.

Depending on the degree of anoxia tolerance the new decreased cytoplasmic pH is stable for some time, but then it drops further due to continued energy shortage which, in concert with a breakdown of transmembrane gradients finally leads to cell death unless the plant finds access to additional energy sources.

The pH falls during fermentation as organic acids are produced and buffering compounds (basic amino acids and primary phosphates) are being consumed. The minimum pH attained during fermentation is a function of original wort pH, wort buffering capacity and the amount of yeast growth throughout fermentation. Lower

beer pH is associated with a lower wort pH, lower wort buffering capacity and increased yeast growth. The pH reaches a minimum of 3.8 to 4.4 before rising slightly toward the end of fermentation (Munroe. 2006). The low pH of the anaerobic wort is unlikely to inhibit the yeasts fermentative capabilities but, may alter fermentation. In future work, worts made from all malts could be fermented and analysed for gravity and ethanol percentage and flavour (low pH may have an adverse effect on product flavour).

4.4. Fermentable sugar content

The total starch levels (% w/w) were higher than expected following 5 d micromalting (figure 3.8), it is possible that the malt flour contained a large proportion of grains with high starch content. The fact that the total starch content of the micromalt decreased from 48 to 120 h micromalting shows that starch solubilisation is taking place during micromalting.

The level of fermentable sugars in the hot water extract is one of the most important parameters in malt specification. Achieving a high level of fermentable sugars in the extract of malt in the brewhouse is a vital part of the economics of industrial processing of malt.

None of the 20 grains analysed from the 5 d aerobic micromalt batch had as high a fermentable sugar level as was obtained by the 20 grains from the 5 d aerobic commercial malt batch (Figure 3.11). These results suggest that micromalting is not as efficient a technique when it comes to maximising fermentability as the malting beds used at Simpsons. It is important to recognise that Simpsons Maltings in Berwick-Upon-Tweed may not be an accurate representative of all British industrial maltings, the study would be more conclusive if 5 d aerobic malt samples had been sourced from a number of maltings.

The range of fermentable sugar content varies between 7.8 mg grain^{-1} in the 5 d aerobic 1 d anaerobic micromalt to 10.5 mg grain^{-1} in the 6 d aerobic micromalt (Table 3.6). These results show that there was surprisingly little variation in the fermentable sugar content of the 20 grains of each malt sample particularly when one considers the

varied distribution values of the enzyme activities analysed. It could be that 20 grains is an insufficient population size to get enough of a spread of data, a higher number of replicas could be analysed. The range in fermentable sugar data may not reflect the large differences in enzyme activity values in individual grains because enzymes may not have access to their substrates due to inadequate proteolytic breakdown of the endosperm, variable endoprotease activity may be indicated. A more complete assessment could have been made if endoprotease activity, β-glucanase activity and levels of β-glucan had also been studied.

The 5 d aerobic commercial malt had a fermentable sugar content of ~ 2 mg grain^{-1} higher than the 5 d aerobic 1 d anaerobic micromalt (Table 3.6), patterns suggest that the higher level of fermentable sugars in the commercial malt can be attributed to higher levels of sucrose and maltotriose (Figure 3.13), these differences were however not statistically significant, a higher population number would need to be analysed for accurate conclusions to be made.

When taking into account the lower mean single grain weight of the 5 d aerobic 1 d anaerobic micromalt (Table 3.2) and calculating the fermentable sugar content mg per litre of wort it becomes apparent that the anaerobic malt has the highest level of fermentable sugars per litre (Figure 3.14) of malts analysed. This is unusual as anoxia is reported to bring about a state of sugar starvation (Felle, 2005). The increased fermentable sugar level in the anoxic grain cannot be due to the lower grain weight of the malt and hence more grains per litre of wort produced as the 5 d aerobic micromalt is of the same weight but, has the lowest amount of fermentable sugars (Table 3.6 and Figure 3.14). This phenomenon must be due to high levels of α-amylase activity (Figures 3.29, 3.30 and 3.31, Table 3.9) and high levels of limit dextrinase activity (Figures 3.45, 3.46 and 3.47, Tables 3.11 and 3.12) in the 5 d aerobic 1 d anaerobic grain.

It is plausible that many of the 5 d aerobic commercial malt grains could have been exposed to periods of anoxia due to insufficient air circulation in the rotating malt beds, which may be a reason why the fermentable sugar level exceeds that of the 5 d aerobic micromalt.

Yeast cells consume glucose and fructose first (Verstren *et al*., 2004), sucrose is broken into glucose and fructose by extracellular invertase and utilised fairly quickly (Menese *et al*., 2002). Maltose is utilised once glucose and fructose reserves have diminished (Novak *et al*., 2004), most yeast cells will metabolise maltotriose as the last sugar (Day *et al*., 2002). The presence of high levels of glucose and fructose in the wort causes the repression of glucanogenesis, the glyoxylate cycle and the uptake of less preferable carbohydrates which can lead to slow or incomplete fermentation, 'off' flavours and poor maintenance of yeast vitality (Verstrepen *et al*., 2004). Fermentable sugar level patterns (Figure 3.13) suggest that there are higher levels of maltose and slightly higher levels of glucose and maltotriose concentrations in the 5 d aerobic commercial malt and 5 d aerobic 1 d anaerobic micromalt when compared to the 5 d aerobic micromalt. Increased levels of glucose and maltotriose are however not significant, higher population sizes would have to be analysed for more conclusive results. High maltose levels in the 5 d aerobic 1 d anaerobic micromalt grains may be due to the enhanced α-amylase activity (Figures 3.29, 3.30 and 3.31). Fermentation could be carried out to see if the higher glucose levels would impede fermentation in the 5 d aerobic commercial malt and the 5 d aerobic 1 d anaerobic micromalt.

4.5. Nitrogen

The nitrogenous material in wort consists of proteins, polypeptides, peptides and amino acids or free amino nitrogen. Much surplus protein is left behind in spent grains and thus wort, when it is oxidised it forms a proteinaceous "scum" which can lead to run off problems. Polypeptides are long chain amino acid sequences of a high molecular weight which contribute to the texture and mouth feel of beer. There are two important groups to consider during brewing: the hydrophobic polypeptides which are responsible for beer foam and the acidic polypeptides which, if not removed can contribute to colloidal instability in beer. Short chain sequences of amino acids (peptides) have a minor effect on mouth feel, texture and body of beer. Free amino nitrogen usually makes up between 10 and 15 % of the total soluble nitrogen and is essential for yeast growth. The level of free amino nitrogen must be above 160 mg l^{-1} as lower levels can lead to defective fermentation (O'Rourke, 2002b). The total soluble nitrogen and free α-amino nitrogen were measured in this investigation, it would have been useful to include the total protein content of 100 individual grains and thus be able to calculate the enzyme activity U per μg protein. The total extractable protein content of grains could have been measured using the Bradford dye binding procedure (Bradford, 1976) described in section 2.8.2.

4.5.1. Total soluble nitrogen

35 to 40 % of malt protein (total nitrogen) is solubilised by the end of mashing this is referred to as total soluble nitrogen in an unboiled wort (O'Rourke, 2002 b).

When the total soluble nitrogen content was measured in wort made from 5 d aerobic micromalt flour made from many grains, it was within the 600 to 900 mg l^{-1} level considered commercially acceptable (O'Rourke, 2002 a) (Figure 3.15). When the total soluble nitrogen content of individual grains from the 5 d aerobic micromalt batch were measured (mg $grain^{-1}$) they were consistently low, particularly compared to individual grains from the 5 d aerobic commercial malt (Figure 3.16). It seems the presence of a few grains with very high total soluble nitrogen content must have been present when the malt flour was prepared thus masking the consistently low total soluble nitrogen levels attained on a single grain basis, although it seems that the 5 d aerobic micromalt is within acceptable parameters this malt is likely to cause brewhouse problems.

When the total soluble nitrogen levels were calculated per litre of wort based on mean single grain values (Table 3.7) and mean single grain weight (Table 3.2) only the 6 d aerobic micromalt was commercially acceptable (Figure 3.19). The 5 d aerobic commercial malt had very high total soluble nitrogen levels which could lead to fining problems. Following calculation the wort made from 5 d aerobic micromalt grains had very low total soluble nitrogen levels per litre, the values obtained suggest insufficient nitrogen for yeast growth. Total soluble nitrogen level differences between the 5 d aerobic micromalt and the commercial malt are indicative of why micromalting is not an accurate depiction of what happens on a commercial scale.

Following 5 d aerobic micromalting the level of total soluble nitrogen appears to increase when incubated for 24 h aerobically (6 d aerobic micromalt) to a commercially acceptable level and decrease when incubated for 24 h anaerobically.

Studies of factors which influence the homogeneity of malt modification suggest that the unmodified grains of a malt sample always contained higher levels of β-glucan and nitrogen than well modified grains (Palmer, 1999; 2000). If this is indeed the case it

could be speculated that the 5 d aerobic commercial malt is under modified while the 5 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt are over modified.

The spectrophotometric method (ASBC, 1992) was used to determine the total soluble protein as it requires very little wort and can be applied to a large number of samples. The method involves diluting a small sample of wort in sodium chloride and measuring the absorbance at 215 and 225 nm. Soluble protein is then predicted using linear regression against a calibration equation developed using a set of Kjeldahl analysis. The 5 d aerobic micromalt has ~ 0.6 mg protein per grain (or ~ 1.5 % soluble protein) (table 3.7). Half of the 5 d aerobic commercially produced malt grains have over ~ 9 mg protein per grain (or over ~ 25 % soluble protein), a value which is unrealistically high. Figure 3.21 shows that the 5 d aerobic micromalt and the 5 d aerobic commercial malt have much similar levels of free amino nitrogen levels than the N data shown in figure 3.16 would suggest. Based upon these findings, it is reasonable to suggest that the spectrophotometric method as described by Haselmore, 1995 and the ASBC, 1992 is not a reliable way to measure total soluble nitrogen.

4.5.2. Free α amino nitrogen

Wort made from 5 d aerobic micromalt homogeneous flour contained 25 mg l^{-1} lower free amino nitrogen content than is stated to be commercially acceptable, low free amino nitrogen content suggests that the wort may be prone to infection by microorganisms (Figure 3.20).

50 % of wort made from the 5 d aerobic commercial grains analysed had higher free amino nitrogen levels than was observed in the wort produced following 5 d aerobic micromalting (Figure 3.21), adding weight to suggestions that micromalting cannot be used as an accurate representative of industrial scale malting. Due to the high calculated levels of total soluble nitrogen in wort made from 5 d aerobic commercial malt (Figure 3.19), the calculated free amino nitrogen content per litre was expected to be higher (Figure 3.24).

Free amino nitrogen levels, calculated using mean single grain values and mean grain weight, are highest in the wort produced using the 5 d aerobic 1 d anaerobic malt

(Figure 3.24). This calculated value of free amino nitrogen in wort made using 5 d aerobic 1 d anaerobic malt was identical to the calculated value of total soluble nitrogen suggesting extensive nitrogen degradation during the 24 h period of anoxia.

The pH of wort made from 5 d aerobic 1 d anaerobic malt will provide favourable conditions for increased peptidase activity and thus increase nitrogen degradation. Endopeptidases break up large protein molecules into relatively large polypeptide chains and have a pH optimum from 3.9 to 5.5. Exopeptidases attack the polypeptide from a specific end stripping off small units to produce amino acids. There are 2 specific exopeptidase groups: carboxypeptidases attack proteins from the carbonyl end and have a pH optimum of 3.9 to 5.5, aminopeptidases attacks proteins from the amino end and have a pH optimum of 4.8 to 5.2 (O'Rourke, 2002 b). Endopeptidases have a pH optimum from 3.9 to 5.5. Proteolytic enzymes are still active at temperatures of 70 ^{o}C so are likely to have survived mashing and be present in the wort. Excessive proteolysis in malting and mashing is not advantageous as it can reduce foam stability (O'Rourke, 2002 b).

4.6. Hydrolytic enzyme activity

Of the starch degrading enzymes it is generally accepted that α-amylase is most abundant and with its resistance to heat is available to hydrolyse not only the malt starch during mashing but, also that of other cereal adjuncts. β-Amylase is thought to be the limiting factor in maximising fermentability due to its lower 'free' activity in comparison to α-amylase and relative thermal instability. Lack of 'free' limit dextrinase activity has also been reported to limit wort fermentability (Bamforth, 2003).

Distribution patterns of α-amylase, β-amylase and limit dextrinase were examined following malting, as these enzymes are the main contributors to starch degradation during malting and mashing and subsequent wort fermentability. The relative distribution of α-glucosidase was not examined in this study as it is of dubious significance in malting and mashing as yeast cells have the ability to utilise maltose, it is important however to recognise that the sugar spectrum created by α-glucosidase activity will undoubtedly have an effect on not only yeast metabolism but also other enzyme activities and as a result should have been studied.

4.6.1. α-Amylase

The α-amylase type A gene (AMY A encoding AMY1 and AMY2) was expressed as early as week 1 post anthesis (Figure 3.25) and throughout malting (Figure 3.26). It is plausible that the increasing α-amylase activity throughout micromalting (Figure 3.27) could be attributed to increased AMY A (encoding AMY1 and AMY2) transcription and translation. It would have been interesting to observe expression patterns of the α-amylase type B gene (AMY B encoding AMY3; Huang *et al*., 1992). Despite isolating the AMY B gene from genomic DNA attempts to clone the gene into the *E.coli* were unsuccessful and as a result a probe for northern blot analysis could not be synthesised, these difficulties could be attributed to a low copy number of the AMY B class in the barley genome. It would have also been useful to observe BASI activity and the effect it has on α-amylase activity.

Type A mRNAs (AMY A) are present in relatively large amounts in unstimulated aleurone cells and increase~ 20 fold following stimulation by gibberellic acid. Type B mRNA (AMY B) is present at very low levels in unstimulated cells but, increases at least 100 fold after cell exposure to gibberellic acid (Rogers, 1985).

The ranges of α-amylase activity in the single grains of the differing malt samples varied from 2.53 in the 5 d aerobic 1 d anaerobic malt to 6.46 mU grain^{-1} in the 5 d aerobic commercial malt (Figures 3.28 and 3.29, Table 3.9). Only one third of dormant α-amylase producing aleurone cells have been found to respond to gibberellic acid stimulus (Schuurink *et al*., 1997). It is hardly surprising that such heterogeneity of α-amylase activity exists on a grain to grain basis when it can also be observed at a cellular level.

When α-amylase activity of the 5 d aerobic micromalt was calculated (mU g^{-1}, Figure 3.31), using mean single grain value (mU grain^{-1}) and mean individual grain weight, it was less than half of what was obtained for the homogeneous flour mixture (Figure 3.27). The flour mixture must have had some grains with high activity in it to conceal those with low activity, if the 100 single grains analysed were ground and the α-amylase activity of the resulting flour measured the result would be much lower.

The α-amylase activity of single grains from the 5 d aerobic 1 d anaerobic malt batch was much higher than the activity of the aerobic malts (Figures 3.29, 3.30 and 3.31, Table 3.9). If some of the commercial malt grains have been subject to periods of anoxia in the malting beds this was not reflected in the α-amylase activity.

This increased α-amylase activity in grains subjected to anaerobic conditions contradicts research by Guglielminetti *et al.* (1995), who showed that in barley overall amylolytic activity was somewhat reduced during anoxia and that α-amylase activity was negligible. The grains used by Guglielminetti were made anoxic throughout germination from imbibition by incubation in nitrogen gas. In this study a somewhat more crude and inexpensive technique which could be easily replicated was employed, grains were allowed to malt for 5 days aerobically and were then packed into an air tight container and incubated for 24 h at 37 ^{o}C. If, like the *Optic* variety used in this study, the α-amylase mRNA of the grains used by Guglielminetti is present on imbibition (Figure 3.25) it is unusual that there was no α-amylase activity in the anoxic grains; this suggests that either anoxic conditions inhibit the translation of α-amylase or that post-transcriptional modification which occurs following the initiation of aerobic germination must play an important part in the activation of α-amylase.

Sugar availability is one of the elements required to support anaerobic metabolism (Perata *et al.*, 1997), in the cereal grain the availability of soluble sugars is limited while starch is stored in large amounts (Loretti *et al.*, 2003). Sugar-mediated modulation of gene expression may be important for plant survival during periods of anoxia. A lack of oxygen causes a shift from mitochondrial respiration to fermentative metabolism (Perata *et al*., 1998). In anoxia tolerant rice cells an increase in glycolytic flux is required to produce sufficient levels of ATP. Strong sugar utilisation through fermentation can result in sugar starvation, as a preventative measure the rice cell induces the synthesis of α-amylase (Perata *et al*., 1997). Rice has a complex multigene family encoding α-amylase. *Amy1A* and *Amy3E* are expressed in normal oxygen levels, in periods of oxygen deficiency they are not expressed but, *Amy3D* is. The *Amy3D* gene encodes for G and H isoforms of α-amylase. *Amy3D* induction is mediated by sensing low carbohydrate concentration and repressed by sugars; induction is totally independent of gibberellic acid hormonal control (Loreti *et al*., 2003). Rice is capable of responding to gibberellins under anoxia but, the response is too slow to explain the rapid production of the α-amylase enzyme (Perata *et al*., 1993). A similar system for survival in anoxic conditions could exist in barley.

When fermentable sugar levels were calculated (mg l^{-1}) using values obtained from mean single grains fermentable sugar levels (mg $grain^{-1}$) and mean individual grain weight, wort made from 5 d aerobic 1 d anaerobic micromalt contained ~10000 mg l^{-1} more than wort made from 6 d aerobic micromalt (Figure 3.14), this difference appears to be due to higher maltose levels in the anaerobic wort (Figure 3.13), which may be due to increased α-amylase activity. Interestingly a similar pattern is observed in the 5 d aerobic commercial malt, it is likely that with poor aeration of malting beds and only partial rotation a proportion of these grains have been subjected to anoxia. It would have been interesting to see if the total fermentable sugar levels had dropped initially in response to anoxic conditions and if a shift in α-amylase activity reflected these low sugar concentrations. While the induction of α-amylase in the aleurone layer will be unaffected by the presence of sugars, the increased maltose levels in the anaerobic malt grains will be repressing α-amylase expression in the embryo (Perata *et al*., 1997).

Under stress conditions animal mitochondria sense metabolic changes e.g. pH, cytoplasmic Ca^{2+}, energy status and reactive oxygen species and respond by releasing cytochrome *c* thus initiating the programmed cell death cascade. In plant cells the presence of all the components which make up the apoptopic cascade remain to be elucidated. In wheat (*Triticum aestrivum L*) root mitochondria, the onset of anoxia caused rapid dissipation of the inner membrane potential, initial shrinkage of the mitochondrial matrix and release of previously accumulated Ca^{2+} (Virolainen *et al*., 2002).

In barley, the group A α-amylases (AMY1 and AMY2) are unaffected by external Ca^{2+} levels, group B α-amylases (AMY3) require Ca^{2+} for synthesis at a step following mRNA accumulation and processing (Deikman and Jones, 1985; Jones and Jacobsen, 1983). Theoretically if barley, like wheat releases previously accumulated Ca^{2+}, this would facilitate the production of a group B α-amylase isozyme AMY3, induced in response to decreased carbohydrate concentration during anoxia.

BASI consists of a 12-stranded β-barrel structure which belongs to the β-trefoil fold family and inhibits AMY2 by preventing access of the substrate to the enzyme active site. The AMY2: BASI complex is characterised by an unusual calcium ion located at the protein: protein interface. Binding of BASI to AMY2 creates a cavity, exposed to the external medium that is ideally shaped to accommodate the extra calcium ion. This

feature may contribute to the inhibitory effect as the key amino acid side chains are in direct contact with water molecules which are in turn ligated to the calcium ion (Vallée *et al*., 1998). When increased from micromolar to millimolar concentrations Ca^{2+} stabilises AMY2: BASI binding (Abe *et al*., 1993) mainly by reducing the rate of complex dissociation. It would have been interesting to know what the Ca^{2+} concentrations were in the anaerobic malt, to have some idea of the stability of the α-amylase: BASI complex. If the AMY2 is complexed to BASI in the anaerobic malt grains, it is possible that a different α-amylase isoform is responsible for increased activity levels.

The later stages of barley grain development have been described as being consistent with hypoxia (Macnicol and Jacobsen, 1992; Gout *et al*., 2001) (section 1.7.2), the endosperm maintains a pH between 4.9 and 5 (Mikola and Virtanen, 1980). Acidic pH has been observed to have an increase on α-amylase synthesis and secretion, hence abundance (Sinjorgo *et al*., 1993) and activity (Fischer *et* al., 1951). The affinity of α-amylase II for BASI is weakened with decreasing pH (Abe *et al*., 1993).

In grain distilling, backset (stillage) from the bottom of the still can be added to the mash in place of water as a water and energy saving measure. At Strathclyde distillery in Glasgow backset is routinely used in place of 40 % of the water used to cook the wheat and is thus present in the mash, lowering mash pH to 4.5 (a pH only 0.19 pH units lower than that of wort made from 5 d aerobic 1 d anaerobic malt, Table 3.4). The addition of backset to a mash caused the α-amylase activity to decrease significantly (Cooper *et al*., 2004), it was speculated that the low pH caused by backset addition was responsible for dramatically decreased α-amylase activity.

Hydrogen peroxide has been detected in plant tissues during anoxia. There was a significant increase in hydrogen peroxide concentration in root meristematic tissue brought about by a mainly enzymatic process due to the trace amount of dissolved oxygen present during anoxia (Blokhina *et al*., 2001). There is a role for thioredoxin in the plant response to oxidative stress (see section 1.6.3). Thioredoxin acts as an electron donor facilitating the removal of hydrogen peroxide via the regulation of GSH-dependent dehydroascorbate reductase and ascorbate peroxidase (Figure 1.10, Wong *et al*., 2003c). Thioredoxin h has been reported to accelerate the appearance of α-

amylase and increase activity (Wong *et al.*, 2002) and control cellular levels of α-amylase by changing the redox status of the BASI inhibitor (Kobrehel *et al*., 1991; Jiao *et al*., 1993; Maeda *et al*., 2003; 2005; 2006). The thioredoxin h that is present in a reduced state in the anaerobic malt grains could be altering the redox status of the BASI inhibitor releasing α-amylase II and increasing activity.

4.6.2. β-amylase

Barley β-amylase is reportedly synthesised during development in a pattern similar to the major storage protein hordein (Giese and Hopp, 1984), it is also synthesised during maturation and desiccation of the grain (Hara-Nishimura *et al*., 1986). Results of northern blots specific for β-amylase show mRNA to be present from 1 week post anthesis and throughout development (Figure 3.32). Northern blots carried out during 1 to 5 d aerobic malting (Figure 3.33) also suggest a presence of β-amylase mRNA; overexposure of the x-ray films however makes these results difficult to interpret.

'Free' β-amylase can form high molecular weight aggregates with itself and other seed proteins (Daussant *et al*., 1981). β-Amylase is extractable from the barley grain as a 'free' fraction which is soluble in aqueous salt solution and a 'bound' fraction, extracted by the addition of a reducing agent in this case L-cysteine, as specified by the Megazyme Betamyl assay kit. β-Amylase is associated with the endosperm protein matrix (Tronier *et al*., 1970) and the starch granule periphery (Hara-Nishimura *et al*., 1986). The release of 'bound' β-amylase by reducing agents suggests that bonding of the enzyme with the protein matrix involved disulphide bonds. During barley grain germination β-amylase is reportedly released by the action of proteases (Sopanen and Lauriere, 1989) and Endopeptidases (Guerin *et al*., 1992). ELISA and immunoblotting procedures have identified a third β-amylase fraction which has been termed 'latent' (Evans *et al*., 1997). In this investigation where the term 'total' β-amylase activity is mentioned it refers to the combined activities of the 'free', 'latent' and 'bound' β-amylase.

Three endosperm specific β-amylase alleles have been identified in cultivated barley at the *Bmy1* locus on chromosome 4 H (*Bmy1*-Sd1, *Bmy1*-Sd2L and *Bmy1*-Sd2H) (Ma *et al*., 2000). The 'free' β-amylase activity U g^{-1} obtained by Evans *et al.* (1997) for *Bmy1*-Sd1 (*Franklin*) and *Bmy1*- Sd2 (*Schooner*) varieties far exceeded the 'free' activity of

the *Optic* variety analysed in this thesis. Interestingly the 'total' β-amylase activity of the *Optic* measured in this thesis was greater than was measured in the *Flanklin* and *Schooner* varieties measured by Evans. The pattern of 'total' activity increase during days 1 to 4 micromalting observed by Evans also occurred in the *Optic* measured during this investigation (Figure 3.34). β-amylase activity in the grain is allele dependent which may account for genotypic differences (Erkkila *et al*., 1998).

As the 'free' β-amylase activity of the *Optic* flour was consistently low from 72 h to 120 h micromalting (Figure 3.34) only the 'total' β-amylase activity was measured in the single grain analysis, a more complete picture of the difference between micromalt and commercially produced malt and the physiological response of a 5 d aerobic micromalt to anoxia, would have been presented had the 'free' β-amylase activity in single grains also been studied. This decrease in 'free' β-amylase activity following 2 d germination can be correlated with β-amylase degradation in green malt due to the activity of serine endoproteinases (Schmitt *et al*., 2007).

When single grain analysis was carried out the β-amylase varied from 0 to ~ 90 mU grain^{-1} in the 6 d aerobic malt (Figure 3.36), from ~ 4 to 60 mU grain^{-1} in the 5 d aerobic commercial malt (Figure 3.35), from ~ 15 to ~ 75 mU grain^{-1} in the 5 d aerobic micromalt (Figure 3.35) and from ~ 5 to ~ 30 mU grain^{-1} in the 5 d aerobic 1 d anaerobic micromalt (Figure 3.36, for cumulative distribution of all malts see Figure 3.37). Significant variation in β-amylase activity has been previously documented and attributed to the grain positioning on a spike (Yin *et al*., 2002). Similar patterns were observed in several cultivars with the grains at the top having the highest activity and grains at the bottom having the lowest activity. The germination time of barley grains was reported to be highest at the top of the spike and lowest in the middle of the spike (Yin *et al*., 2001) suggesting that β-amylase activity is independent of germination rate and hence grain weight (see section 4.2), there is little documented evidence to suggest that β-amylase is synthesised during germination. It would have been useful to investigate the relationship between the grain positioning on a spike and all of the parameters examined for the purposes of this thesis with aims of maximising brewhouse performance.

In single grains the highest mean 'total' β-amylase activity is observed in grains from the 5 d aerobic micromalt (Figure 3.35, Table 3.10) and the lowest mean β-amylase activity in the 5 d aerobic 1 d anaerobic micromalt (Figure 3.36, Table 3.10).

When barley grains were germinated anaerobically under nitrogen gas, β-amylase activity was higher than in grains germinated in aerobic conditions (Guglielminetti *et al*., 1995), it was also found that β-amylase expression was up-regulated in response to anoxia. It appears that the barley grains act differently to onset of anoxia at imbibition than they do following 5 d aerobic germination.

Synthesis of β-amylase during grain development is dependent on and regulated by nitrogen nutrition (Giese *et al*., 1984). A positive correlation between β-amylase and protein content in grains has been established (Arends *et al*., 1995; Santos *et al*., 1996; Wang *et al,*. 2003). Single grain analysis of the 5 d aerobic 1 d anaerobic micromalt had lower levels of total soluble nitrogen (mg grain^{-1}, Figures 3.17, 3.18 and 3.19) and β-amylase activities (mU grain^{-1}, Figures 3.36, 3.37 and 3.38) than the other malts analysed, the anaerobic malt also had high levels of free amino nitrogen (Figures 3.22, 3.23 and 3.24). High β-amylase activity was measured in single grains from the 5 d aerobic micromalt batch (Figures 3.35, 3.37 and 3.38); this was accompanied with low total soluble nitrogen content (Figure 3.16) and free amino nitrogen content (Figure 3.21). The increased free amino nitrogen level coupled to decreased total soluble nitrogen content and β-amylase activity found in the anaerobic but, not aerobic micromalt speculates that the β-amylase enzyme is itself being degraded during anoxia.

In this investigation the decrease in β-amylase activity in response to anoxia is coupled to an increase in α-amylase activity. Furthermore the α-amylase/trypsin inhibitor BASI has been reported to inactivate the malt serine endopeptidase SEP-1 (Jones *et al*., 2003); these findings could indicate that the inactivation of BASI in the anaerobic malt has not only activated α-amylase but also the serine endoproteinase which could be responsible for the degradation of β-amylase (Schmitt *et al*., 2007).

β-amylase was shown to be activated by the cysteine endoprotease EP-B (Guerin *et al*., 1992). It is logical that for activity the cysteine residues localised at the active site

of cysteine proteinases must be reduced. Hydrogen peroxide increases due to anoxia (Blokhina *et al*., 2001) would inevitably act as an oxidising agent thus inhibiting the activity of cysteine endoproteases and hence the activation of β-amylase. This theory is supported by observations on the inhibition of cysteine endoprotease activity by hydrogen peroxide (Pöyri *et al*., 2002).

Cyclodextrins make up a family of cyclic oligosaccharides typically containing a number of glucose monomers ranging from 6 to 8 units in a ring creating a cone shape. α-Cyclodextrin consists of 6 glucopyranose units while β-cyclodextrin consists of 7 glucopyranose units. Cyclodextrins and maltose inhibit the activity of β-amylase by binding to the active cleft (Mikami *et al*., 1993). The 3-D structure of soybean β-amylase complexed with α-cyclodextrin indicates that 2 glucose units of the α-cyclodextrin molecule interact with the entrance of the β-amylase active site cleft thus, physically blocking the entrance to the active site (Wong *et al*., 2003b). The binding of β-amylase to β-cyclodextrin occurred at the same two glucose binding sites as the α-cyclodextrin (Adachi *et al*., 1998). Cyclodextrins are produced from starch via enzymatic conversion involving cyclodextrin glycosyltransferase and also α-amylase (Biwer *et al*., 2002). The increased α-amylase activity observed in the anaerobic malt could have led to an increase in cyclodextrin concentration thus inhibiting the β-amylase activity. To confirm or reject this theory, HPLC would have to be carried out in order to detect cyclodextrin abundance. An increased concentration of cyclodextrins would be unexpected in the 5 d aerobic 1 d anaerobic micromalt as β-cyclodextrins are also inhibitors of limit dextrinase (Kristensen *et al*., 1998) and limit dextrinase activity is enhanced by the anoxic conditions (Figures 3.45, 3.46 and 3.47).

The addition of backset to wheat mashes resulted in a loss of β-amylase activity (Cooper *et al*., 2004). Backset addition lowers the pH of the mash to ~ 4.5, a pH similar to that observed in the wort made from the 5 d aerobic 1 d anaerobic malt. It is unlikely that the pH of the wort made from the 5 d aerobic 1 d anaerobic malt is responsible in decreased activity as β-amylase has a broad pH spectrum ranging from 4.0 to 7.5 (Lundgard and Svensson, 1987).

4.6.3. Limit dextrinase

Limit dextrinase activity and mRNA have been reported in very low levels in developing grains (Sissons *et* al., 1993; Burton *et al*., 1999). Limit dextrinase mRNA levels, enzyme abundance and activity increase significantly during germination and in response to gibberellic acid (Lee *et al*., 1984; Longstaff *et al*., 1993; Kristensen *et al*., 1993; MacGregor *et al*., 1994b; Schroeder *et al*., 1998; Kristensen *et al*., 1998; Burton *et al*., 1999). Northern blot analysis shows limit dextrinase mRNA to be produced from 1 to 5 d germination, with most limit dextrinase transcript being present on day 2 (Figure 3.39).

Limit dextrinase exists in an inactive 'bound' form and an active 'free' form. The 'bound' form is thought to be complexed with a proteinaceous inhibitor protein and can be released by treatment with reducing agents DTT and cysteine (Lee and Pyler, 1984; Longstaff *et al*., 1991; Sissons *et al*., 1994; 1996). In this investigation 'total' limit dextrinase activity refers to the 'bound' and 'free' limit dextrinase activities and is determined following extraction in the presence of DTT as is specified by the limit dextrizyme assay kit provided by Megazyme (see section 2.5.6).

Figure 3.40 illustrates the increases in 'free' and 'total' limit dextrinase activity of 5 d aerobic micromalt homogeneous flour during the first 120 h malting. 'Total' limit dextrinase activity of the *Optic* used in this investigation was comparable to that obtained in *Golden promise* measured by M^{c}Cafferty *et al*. 2000, the 'free' limit dextrinase activity of the *Optic* was ~ 100 mU g^{-1} higher than in the *Golden promise*.

Genotypic variation in 'free' limit dextrinase activity has been previously observed (Lee *et al*., 1984; Longstaff *et al*., 1993). An ELISA was developed using limit dextrinase specific antibodies to measure activity in malt flour extracts, malts prepared from barley cultivars of wide genetic diversity showed a threefold variation in 'total' activity (Sissons *et al*., 1992). For the purposes of this thesis only the cultivar *Optic* was used and the limit dextrizyme assay kit supplied by Megazyme was utilised, large variations between grains of the same malt batches were observed. The 'free' limit dextrinase activity of single grains varied between 0 mU grain^{-1} in the 5 and 6 d aerobic micromalts to ~15 mU grain^{-1} in the 6 d aerobic micromalt (Figures 3.41, 3.43 and 3.46; Table 3.11). The 'total' limit dextrinase activity of single grains varied form ~ 4 mU grain^{-1} in the 5 d

aerobic commercial malt and micromalt to ~ 18 mU grain^{-1} in the 6 d aerobic and 5 d aerobic 1 d anaerobic micromalts (Figures 3.42, 3.44 and 3.45., Table 3.12).

When limit dextrinase activity of single malt grains was measured (as described in section 2.6.6), the extraction time was 5 h. It is possible that 5 h was insufficient time to release all of the limit dextrinase from the flour. Longer extraction times could have been carried out to ensure full extraction of the enzyme. For comparative purposes all grains from malt sample analysed were treated in the same way.

Limit dextrinase production can be influenced by germination/malting conditions. For example barley which was continuously hydrated throughout germination was found to have higher limit dextrinase levels than malt (Longstaff *et al*., 1993; Kristensen *et al*., 1993; 1998). As the 5 d aerobic micromalt, the 6 d aerobic micromalt and 5 d aerobic 1 d anaerobic micromalt were all prepared at Heriot-Watt micromaltings, variations in limit dextrinase activities between the batches cannot be attributed to differing malting conditions.

When limit dextrinase activity was measured in single grains it was established that the highest mean 'free' and 'total' activities were from the 5 d aerobic 1 d anaerobic malt batch and the lowest from the 5 d aerobic micromalt and 5 d aerobic commercial malt (Figures 3.41 to 3.45., Tables 3.11 and 3.12).

When barley variety *Golden promise* was subjected to aerobic followed by anaerobic germination conditions (as based upon Dixon's enzymic malt, patent 582423, 1956), the malt produced had a higher 'total' limit dextrinase activity, a higher proportion of which was 'free' (M^{c}Cafferty *et al*., 2000). 'Total' limit dextrinase activity reached a maximum in the *Golden promise* following 6 d aerobic, 3 to 4 days anaerobic germination. Unfortunately due to silage-type aroma and low nitrogen levels this malt is unsuitable as a substrate for industrial brewing and when added to an aerobically produced malt, the 'free' limit dextrinase activity was inhibited. When *Optic* was micromalted for 5 d aerobically and 1 d anaerobically the silage-type aroma was not present and the 'free' and 'total' limit dextrinase activities were enhanced (Figures 3.45, 3.46 and 3.47., Tables 3.11 and 3.12).

When 'total' limit dextrinase was extracted from barley in the presence of reducing agents during germination in aerated and anoxic conditions; activity was only present in the aerobic grains (Guglielminetti *et al*., 1995). As limit dextrinase mRNA has been reportedly present at maturation (Burton *et* al., 1999) some activity was to be expected, but as no limit dextrinase activity was observed by Guglielminetti it can be postulated that as for α-amylase, either the anoxic conditions from imbibition have inhibited limit dextrinase translation or that post-translational modification which occurs following the initiation of aerobic germination must be important for the activation of limit dextrinase.

When thioredoxin h was over expressed in transgenic barley, enhanced limit dextrinase activity was observed (Cho *et al*., 1999). When recombinant limit dextrinase from maize (ZPU1) was over expressed in *E.coli* it was activated by treatment with thioredoxin h (Wu *et al*., 2002). The thioredoxin h, present in reduced state in the anaerobic malt grains in response to hydrogen peroxide induced oxidative stress, could be altering the redox status of the limit dextrinase inhibitor releasing limit dextrinase and increasing activity. When Eisner *et al*., 2008 attempted to release limit dextrinase in an active form using the thioredoxin system of *Escherichia coli* they were unable to do so.

The limit dextrinase activity of an extract containing both limit dextrinase and its proteinaceous inhibitor was greater following extraction and assay at pH 4.4 than it was at pH 5.5 (Bryce *et al*., 2004). Limit dextrinase activity was low during mashing and activated during fermentation. The activation of limit dextrinase occurred when the wort pH decreased. This decreased pH occurs naturally during fermentation (Munroe, 2006). Furthermore the 'free' limit dextrinase activity was found to increase significantly when 40 % of the wheat cook water was replaced with backset, which lowered the mash pH 4.5 (Cooper *et al*., 2004). A threefold increase in limit dextrinase activity was also observed when extraction and assay pH were lowered to 4.0 (Heisner *et al*., 2008). At pHs below 5.0, where limit dextrinase is below its optimum pH for activity, activity is increased due to the dissociation of the enzyme: inhibitor complex. The protection of limit dextrinase from mashing temperatures afforded by the limit dextrinase inhibitor is lost at these lower pHs (M^{c}Cafferty *et al*., 2004). Results of this investigation suggest that the low pH of the 5 d aerobic 1 d anaerobic wort (Table 3.4) could be responsible for dissociating the limit dextrinase: limit dextrinase inhibitor complex causing activity of limit dextrinase to increase, if this is indeed the case the limit dextrinase could be sensitive to high mashing temperatures and hence not survive

mashing, the absence of limit dextrinase activity during fermentation associated with decreasing pH could lead to an altered ethanol percentage.

The 5 d aerobic commercial malt had the lowest mean 'total' limit dextrinase activity, but a higher proportion of which was 'free' than in any other malt analysed (Figures 3.41 and 3.42., Tables 3.11 and 3.12). Irrespective of mean 'total' activity the mean 'free' activity of the commercial malt exceeded that of the 5 d aerobic micromalt. If some of the commercial grains were subjected to periods of anoxia in the malting beds this has not been reflected in the limit dextrinase activity.

The mature barley limit dextrinase consists of a single polypeptide chain of 884 amino acid residues with a calculated molecular mass of 97.42 kDa (Burton *et al*., 1999). SDS-PAGE analysis reported a molecular mass of 103 to 105 kDa and a pI of 4.2 to 4.6 (Sissons *et al*., 1992; MacGregor *et al*., 1994b; Kristensen *et al*., 1998). As seven potential N-glycosylation sites exist in the limit dextrinase amino acid sequence, the difference in calculated and deduced molecular weight can be attributed to alternative splicing or glycosylation. When limit dextrinase was partially purified and the extract was run on SDS-PAGE (Figure 3.49) a band was present above 83 kDa, this was assumed to be the limit dextrinase protein.

4.7. Limit dextrinase inhibitor

Barley contains two isoforms of a potent limit dextrinase inhibitor, designated as low and high pI forms (MacGregor *et al*., 2003). Limit dextrinase inhibitor was shown to be of importance in the regulation of limit dextrinase activity, not only during germination but also during starch synthesis (Stahl *et al*., 2004).

Protein extracts made from malt following steeping were found to inhibit exogenous partially purified limit dextrinase activity by > 80 % (Ross *et al*., 2003). In this study the amount of limit dextrinase inhibitor present in 50 µl of crude extract prepared from mature grain was sufficient to inhibit a mean value of 63.77 $\pm$ 12.64 (Table 3.13), a variation between 40 and 100 %, of 50 µl of partially purified limit dextrinase with an activity of 5 mU (Figures 3.50 and 3.51). The steeping regime utilised by Ross *et al.* may have resulted in increased inhibitor levels, the expression of the *Optic* limit

dextrinase inhibitor could be examined to see if it is expressed during malting. It is also possible that the partially prepared limit dextrinase Ross *et al.* used had a lower activity value than 5 mU or that an inadequate number of replicates were analysed by Ross *et al.* to see the true spread of data.

During this investigation attempts were made to purify limit dextrinase inhibitor protein from a crude extract of mature barley. Crude extract was heated prior to being passed through a CM Sepharose column and then either a Mono Q column or gel filtration column (see section 2.8.5), on each occasion the recovery (%), the total protein (mg) and the specific activity (AU μg^{-1}) were repeatedly low in comparison to what was obtained by MacGregor *et al.* (1994), despite using similar protocol.

To further analyse *in vivo* properties of the limit dextrinase inhibitor crude grain extract was run through a pre-calibrated gel filtration column. Subsequent SDS-PAGE and western blot analysis (using low pI limit dextrinase inhibitor specific antibodies) of fractions revealed that the low pI limit dextrinase inhibitor of the mature *Optic* barley was present in high molecular weight fractions between 66.2 kDa and 2000 kDa (Figures 3.53 and 3.54) when it was expected to be present in fractions 12 and 13 as the low pI limit dextrinase inhibitor is 12.928 kDa (MacGregor *et al*., 2000) and cytochrome c (mass 12.4 kDa) eluted from the column at fraction 13. Thus it appears that the inhibitor is present as a complex, possibly together with other proteins such as limit dextrinase.

As northern blots showed limit dextrinase mRNA transcripts to be present at maturation (Figure 3.39) it is plausible that limit dextrinase will already be bound to the limit dextrinase inhibitor at this stage. The purified limit dextrinase has a molecular weight of 104 kDa (Sissons *et al*., 1992) and the low pI limit dextrinase inhibitor has a molecular weight of 12.928 kDa (MacGregor *et al*., 2000) coupled to the fact that the enzyme: inhibitor complex has a 1:1 molar ratio, which is unaltered in the presence of excess enzyme or inhibitor (MacGregor *et al*., 2003), confirms that the likelihood of this complex eluting into fraction 2 is improbable as proteins present in fraction 2 are likely to have a higher molecular weight than 116 kDa (calculated molecular weight of limit dextrinase: limit dextrinase inhibitor complex) .

As well as binding to limit dextrinase in mature barley grains, the low pI limit dextrinase could be binding to other proteins creating a complex or aggregating with itself forming a high molecular weight molecule.

The limit dextrinase inhibitor gene is expressed at 2 to 4 weeks post anthesis in the endosperm. The limit dextrinase inhibitor protein is present and active from 4 weeks post anthesis where it accumulates in the outer region of the starchy endosperm, which coincides with the region where small B-type granules are found (Stahl *et al*., 2004; Stahl *et al*., 2007).

14-3-3 proteins are known to act as scaffold proteins (Comparot *et* al., 2003) (see section 1.6.3). The limit dextrinase inhibitor sequence contains a 14-3-3 protein binding motif and binds to 14-3-3A and 14-3-3C proteins in a phosphorylation dependent manner (Stahl *et al*., 2007). Proteomic analysis of 14-3-3 binding proteins from developing barley grains revealed that several enzymes involved in starch metabolism contained 14-3-3 binding motifs including α-amylase, the α-amylase inhibitor and β-amylase (Alexander *et al*., 2006). Furthermore when the limit dextrinase inhibitor gene was down regulated, increases in the 'free' limit dextrinase activity observed was accompanied by unexpected pleiotropic effects on α-amylase, β-amylase and starch synthase (Stahl *et al*., 2004).

Despite having never been observed in plants thus far, there is a possibility that binding to 14-3-3 dimers with multiple binding sites may result in bringing different proteins together. In many cellular processes polypeptides are associated to one another or to subcellular structures, small molecules or nucleic acids. Mutational analysis has shown that starch hydrolytic and biosynthetic enzymes are closely linked (see section 1.6.4).

Physical interactions between starch branching enzymes and starch synthases have been identified from the endosperm amyloplast of developing wheat (Tetlow *et al*., 2008). The formation of these starch branching enzyme: starch synthase complexes are phosphorylation dependent and the enzymes are active. *In vivo* protein – protein interactions between starch synthases and starch branching enzymes have also been found in maize (Hennen-Bierwagen *et al*., 2008).

Aerobic levels of phosphorylation are maintained during the first 2 h anoxia, after which they decrease, by 48 h anaerobiosis most protein exists in unphosphorylated form (Subbaiah *et al*., 2001). If the complex containing the limit dextrinase inhibitor protein exists following 5 d aerobic germination and involves association by 14-3-3 proteins (and is hence phosphorylation dependent), abundance of the complex will decline with the phosphorylation status.

There is a possibility that starch hydrolysis may operate using complex protein interactions, as well as starch biosynthesis.

4.8. Further investigation

- As individual grain germination rate, weight and β-amylase activity have been reportedly affected by their position on the barley spike it would be advantageous to the brewer to determine the position on the spike which produces the best performing grain. The nitrogen content, fermentable sugar concentration and enzyme activity could be measured in single grains taken from differing spike positions of several cultivars.

- To conclusively confirm or reject suggestions that a proportion of the 5 d aerobic commercial malt was anoxic, it would be useful to take samples from different depths of a malting bed and carry out single grain analysis on the grain weight, nitrogen content, fermentable sugar content and hydrolytic enzyme activity.

- Fermentation of the malt samples used in this investigation could be carried out to confirm or disprove suspicions that the commercial malt will perform better in the brewhouse than the 5 d aerobic micromalt and that 1 d anaerobiosis following 5 d aerobic micromalting will produce different ethanol yields to aerobic counterparts.

- To find out if the limit dextrinase inhibitor protein is aggregating with itself or if it is part of a multiprotein complex further work should be carried out. A native PAGE could be carried out whereby complexed proteins would not dissociate, bands could be removed and proteins purified. The purified protein of individual bands could then be run on an SDS-PAGE whereby complexes would be dissociated, western blots using antibodies specific for the low pI limit dextrinase inhibitor would allow the band of complexed protein containing the

limit dextrinase inhibitor to be identified. The native PAGE could then be repeated, the band containing the limit dextrinase inhibitor protein removed, the protein complex could then be purified from the gel and run on a 2-D PAGE. 2-D PAGE allows proteins to be separated according to charge (pI) by isoelectric focussing in the first dimension and size (Mr) by SDS-PAGE in the second dimension. 2-D PAGE has a unique capacity for the resolution of complex mixtures of proteins (Görg *et al*., 2000). 2-D PAGE has become the most important technology for high resolution separation of protein for proteomics (Fey *et al*., 2001). 2-D PAGE results in a gel with proteins spread on its surface. Proteins can then be excised and digested with proteolytic enzymes e.g. trypsin. The peptide combination can then be pooled with a laser absorbing matrix and loaded into the MALDI-TOF MS spectrometer where a laser is focused towards the sample matrix combination. The matrix will absorb energy and ionise the peptide and release it. The ionisation process imparts only a single charge on the peptides. The liberated peptide then travels through a flight tube prior to being detected by a detector. Since the charge is unchanged the time of flight will depend on the mass of the peptides. \these are generated for a particular protein and remain the benchmark for that particular protein, they can be recognised as peptide mass fingerprints (PMFs). The PMFs are stored in a database and associated to theoretically digested proteins from various other databases by an in built software programme e.g. MASCOT and homology database Basic Local Alignment Tool (BLAST). The programme then identifies the protein (Beranova-Giorgianni, 2003).

Appendix A PCR fragment cDNA sequences and primer sequences

β- Amylase PCR fragment from accession no. AF414081 bamy1 cDNA

taacgatgtcgggcagtacaatgacactcccgagagaactcaattcttcagagacaacgggacatacctaag
tgagaaggggaggtttttccttgcatggtactccaacaatctgatcaagcacggtgacaggatcttggatgaag
caaacaaggtcttcttgggatacaaggtgcaactggcaatcaagatctctggcattcactggtggtacaaggtt
ccaagccatgcagccgagct

cacagctgggtactacaacttacatgatagagacggctacagaaccatagcacgcatgctcaaaaggcacc
gtgctagcattaacttcacttgcgcggagatgagggattcggagcaaagctcgcaggcgatgagcgcacca
gaagaactagtccaacaggtgttgagtgctggatggagagagggcctaaatgtggcatgcgaaaacgcgct
tccacgatatgatccaactgcttacaacaccatactcaggaatgcgaggcctcatggaatcaaccagagcgg
ccctcctgagcacaagctgtttggattcacctaccttcggctgtcgaatcagctggtggagggacaaaactatg
cc

β- Amylase primer pair

BA-F

taa cga tgt cgg gca gta ca

BA-R

ggc ata gtt ttg tcc ctc ca

α- Amylase PCR fragment from accession no. BLYAMYAA type A isozyme cDNA

atctggtgaactgggtggacaaggtgggcggcgcggcctcggcaggcatggtgttcgacttcacgaccaaa
gggatactgaacgctgccgtggagggcgagctgtggaggctgatcgacccgcaggggaaggcccccggc
gtgatgggatggtggccggccaaggccgccaccttcgtcgacaaccacgatacaggctccacgcaggccat
gtggccattcccctccgacaaggtcatgcagggctacgcgtacatcctcacccaccccggcatcccatgcatc
ttctacgaccatttcttcaactgggggtttaaggaccagatcgcggcgctggtggcgatcaggaagcgcaacg
gcatcacggcgacgagcgctctgaagatcctcatgcacgaaggagatgcctacgtcgccgagatagacgg
caaggtggtggtgaagatcgggtccaggtacgacgtcggggcggtgatcccggccgggttcgtgacctcggc
acacggcaacgactacgccgtctgggagaagaacggtgccgcggcaacactacaacggagctgaagtct
gcactgatccgtcattcgatcgagcatgaa

α- Amylase primer pair

amyA-F

atc tgg tga act ggg tgg ac

amyA-R

ttc atg ctc gat cga atg ac

Limit dextrinase PCR fragment from accession no. AF122049 limit dextrinase cDNA

gaatggagcacctacggaaattatctgatgctggtttgactcatgtgcatttgttgccaagctttcattttgctggcgt
tgacgacattaagagcaactggaaatttgtcgatgagtgtgaactagcaacattccctccagggtcagatatgc
aacaagcagcagtagtagctattcaggaagaggacccttataattgggggtataaccctgtgctctgggggttt
ccaaaaggaagctatgcaagtgaccctgatggcccgagtcgaattattgaatatcgtcagatggtccaggccc
tcaatcgcataggtcttcgtgttgtcatggatgttgtatacaatcatctagactcaagtggcccctgcggtatcagct
cagtgcttgacaagattgttcctgggtactatgttagaagggatactaatggccagattgagaacagtgcagct
atgaacaatacagcaagtgagcatttcatggttgataggttaatcgtggatgacctttgaactgggcagtaaac
tacaaagttgacgggttcagatttgatcttatgggccatatcatgaaacgcacaatgatgagagcaaaatctgct
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gctgaagttgcacgcaatcaacgtggaataaatgggtcccagcttaatatgagtggaacggggattggtagct
tcaatgatagaatccgggatgctattaatgggggtaatccctttggtaatccgct

Limit dextrinase primer pair

LD-F

gaa tgg agc acc tac gga aa

LD-R

agc gga tta cca aag gga tt

M13 forward and M13 reverse primer pair

M13F

cag gaa aca gct atg acc

M13R

gtt ttc cca gtc acg acg

Appendix B Home pages of suppliers

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Published papers

Limit Dextrinase – Does Its Malt Activity Relate to Its Activity During Brewing?

Calum A. McCafferty[1], Helen R. Jenkinson[1], James M. Brosnan[2] and James H. Bryce[1,3]

ABSTRACT

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Limit dextrinase (EC 3.2.1.142) hydrolyses α-1,6 glucosidic bonds in amylopectin and branched dextrins. Measurement of limit dextrinase activity during fermentation of unboiled wort at the pH of the wort has shown that its activity increases almost 10 fold during the first 10–15 h of fermentation and this increase in activity is unaffected by the presence of leupeptin, a cysteine protease inhibitor. The increase in activity seen when assays were carried out at pH 5.5 was much smaller and was reduced by leupeptin. The activity of limit dextrinase declined slowly during the latter part of the fermentation. It was established that the optimum pH for rapid extraction and assay of malt limit dextrinase in the absence of a reducing agent is approximately 4.5, but in the presence of dithiothreitol, at pH 5.5, activities 2–3 times higher can be obtained after 5 h extraction (600–700 mU/g dry weight). Limit dextrinase activities after 1 h extraction at mashing temperatures were below 20 mU/g dry weight if the mash pH was below 5.0. It is concluded that at pHs below 5.0, where limit dextrinase activity is below its optimum for activity, limit dextrinase activity increases due to dissociation of the inhibitor/enzyme complex. The protection from mashing temperatures of 65°C afforded by the inhibitor is lost at these lower pHs.

Key words: Brewing, distilling, limit dextrinase, malting.

INTRODUCTION

Limit dextrinase (EC 3.2.1.142) degrades (α-1,6 glucosidic bonds in amylopectin and branched dextrins[4,20,24]. This malt enzyme is important in the brewing and distilling industries because it has the potential to convert non-fermentable dextrins to fermentable sugars. Promoting limit dextrinase activity will thus lead to altered wort carbohydrate compositions with increased fermentability[33]. In the barley endosperm, starch mobilisation is carried out by the combined action of an array of hydrolytic enzymes. Starch mobilisation in germinating barley grains is initially catalysed by α-amylase, and the resulting dextrins are further hydrolysed by limit dextrinase, β-amylase and α-glucosidase[9,18]. These enzymes also degrade starch and dextrins during mashing following extraction of starch degradation products and enzymes into wort.

In mature barley grains, limit dextrinase activity is minimal[19] but during germination activity slowly increases[14,15,32]. Limit dextrinase has been shown to exist in free (soluble, active), latent (soluble, inactive) and bound forms in germinating barley[13,34] and its conversion to the free soluble form has been extensively studied[17]. Limit dextrinase is converted from an inhibited form to an active form during germination most probably via proteolytic activation[15] or through thioredoxin linked reduction[5]. Malt often has a free enzyme activity making up less than 20% of the potential total activity[14], although activation of free enzyme approaching 35% has been found in barley malts of variety Static and Chariot[31]. In malt, free limit dextrinase activity was increased above these levels by germinating grains under anaerobic (reducing) conditions, an environment which could have increased the activity of cysteine proteases[3,25], which may have degraded the limit dextrinase inhibitor. Anaerobically germinated grain, following a period of normal malting, produced grains containing a free limit dextrinase activity constituting over 80% of the total limit dextrinase activity. Thus it is possible to produce malt with high free limit dextrinase activity.

Wort is not boiled prior to distillery fermentations, as it is during brewing, enabling hydrolytic enzymes that survive mashing temperatures to be present and active during the fermentation process[34,36]. Non-fermentable limit dextrins can constitute as much as 25% of the carbohydrate in wort[7]. These dextrins may contribute to the mouthfeel of beer but will add no contribution to flavour, texture or body of distilled products. Limit dextrinase survives mashing and has been shown to be important during fermentation where it reduces the percentage of branched dextrins present thus increasing wort fermentability[2,34,36].

Proteinaceous limit dextrinase inhibitor(s) have been demonstrated in malt and are affected by malting conditions[16,17,23]. Mashing and fermentation conditions may also have an effect on limit dextrinase inhibitor, limit dextrinase activity and its activating agents (cysteine proteases) due to the changes in temperature, pH and oxidation con-

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ditions that occur during these processes. Indeed, it has been suggested that limit dextrinase-inhibitor complexes could dissociate as the pH drops during fermentation[31]. The effectiveness of inhibition varies with pH, the strongest degree of inhibition was found to occur at pH 5.5 and the weakest at pHs of 4.5 and 8.0[16,21].

The aim of this study was to determine the changes that occur in limit dextrinase activity during fermentation and find out why these changes occur. Limit dextrinase activity was therefore determined during fermentation, not only after adjustment to pH 5.5 for assay, as was carried out by Bringhirst et al.[2] but also at the pH of the fermentation, and also following acidification of wort pHs.

In this paper, we provide further evidence that when limit dextrinase is extracted from malt using conventional procedures at pH 5.5, its activity is reduced by its proteinaceous inhibitor and that enhancement of this activity is due to proteolytic activation in the presence of DTT. However, at pHs below 5, the inhibitor looses its effect on limit dextrinase, leading to an increase in limit dextrinase activity without the need for proteolytic activation. At these lower pHs the activity is however suboptimal compared to the activity attained at pH 5.5 if the inhibitor was proteolytically degraded.

MATERIALS AND METHODS

Chemicals and biological materials

Barley *Hordeum vulgare* (variety Golden Promise) was supplied by Moray Firth Maltings, Arbroath, Scotland. Chemicals were obtained from the Sigma Chemical Company, Poole, Dorset, England or BHD, Leicestershire, England and were of analytical or the highest grade available. Limit Dextrizyme Kits were from Megazyme International, Bray, Wicklow, Ireland. Distillers yeast 'M' strain was obtained from Quest International, UK.

Malt production

Normal aerobic germination was carried out using a Seeger micromalting plant[4,25]. Anaerobic germination was based on Dixon's enzymic malt (patent 582,423, November 1946) and achieved by tightly packing partially malted grains into 60 mL plastic containers, which were then sealed and incubated at 37°C. Malts used in experiments, unless otherwise indicted, were produced in the micromaltings. Aerobic malt was germinated for 5 d, anaerobic malt was germinated for 5 d aerobically in the micromaltings and 6 d anaerobically (a total of 11 d), malts were kilned at 50°C for 24 h[25].

Limit dextrinase extraction and assay

Limit dextrinase of malts was extracted and assayed in 100 mM sodium maleate buffer as specified by the Limit Dextrizyme method[26]. Ground malt was sealed in 50 mL Oak Ridge centrifuge tubes and allowed to shake in an incubator at 40°C for 5 h or as stated in results section text. Free activity is the activity measured after extraction for 5 h without DTT, and total activity is measured after 5 h extraction with DTT. Assay of mash or wort limit dextrinase activity was done without further extraction. If the pH of mash or wort was adjusted, the adjustment was done with NaOH or HCl at 20°C. Where limit dextrinase activities were measured with different extraction procedures (buffering, pH, length of extraction), the term 'free' in quotes indicates that DTT was not present, and 'total' in quotes indicates that DTT was present. Assays were carried out using azurine cross-linked pullulan tablets from Megazyme International. The aerobically germinated malt, after 5 h extraction, had a 'free' limit dextrinase activity of 45 mU/g and a 'total' activity of 506 mU/g. Anaerobic malt, 5 d aerobic germination followed by 6 d anaerobic germination, had a 'free' activity of 500 mU/g, a value identical to the 'total' limit dextrinase activity of the aerobically germinated malt.

Fermentations

Mash was made by adding 100 g of 6 d aerobic malt, ground with a Bühler Miag mill set to 0.2 mm, to 360 ml water at 65°C for 1 h, the final volume was adjusted to 500 ml. All other aspects of mashing adhered to standard Institute of Brewing methods[11,35]. Standard Institute of Brewing fermentation methods were used whereby 1.25 g yeast (Distillers yeast, 'M' strain, Quest International, UK) was suspended in 250 ml filtered wort, fermentations were set up in 250 ml conical flasks containing 90 ml wort. The flasks were stoppered with fermentation locks (Boots, UK) and incubated at 33°C for 44 h.

Leupeptin

Leupeptin is an inhibitor of cysteine protease activity[15]. A 360 µL stock solution of leupeptin was added to the 250 mL conical flasks containing 90 mL wort to give a final concentration of 0.008% (w/v). The limit dextrinase activity of wort with leupeptin in the presence of DTT was determined. Activities were compared to values obtained in standard fermentations of the wort, and also to a control wort with DTT in the presence and absence of yeast.

RESULTS

The effects of the cysteine protease inhibitor, leupeptin, and pH on limit dextrinase activities during fermentation

Leupeptin was added to all malt wort at a concentration of 0.008%, sufficient to prevent the activation of limit dextrinase by cysteine proteases[15]. Limit dextrinase activity and pH were measured throughout the subsequent fermentation to discover how limit dextrinase activity changed and how this activity was affected by the pH. A control fermentation was carried out with no addition of leupeptin. Limit dextrinase activity was measured after adjusting the wort pH to 5.5 (see materials and methods section) and also directly at the pH of the wort. The control fermentation shown in Fig. 1, revealed a large increase in limit dextrinase activity by 7 h that appeared to be associated with the decreases in fermentation pH below 5. In both the control fermentation (Fig. 1) and the fermentation with added leupeptin (Fig. 2) a decrease in fermentation pH from 5.5 to 4 was observed. Limit dextrinase activity of the wort measured as the fermentation decreased to a value ranging from ca. 4.4 to 4.2. This activity compares with the malt's free activity of 45 mU/g

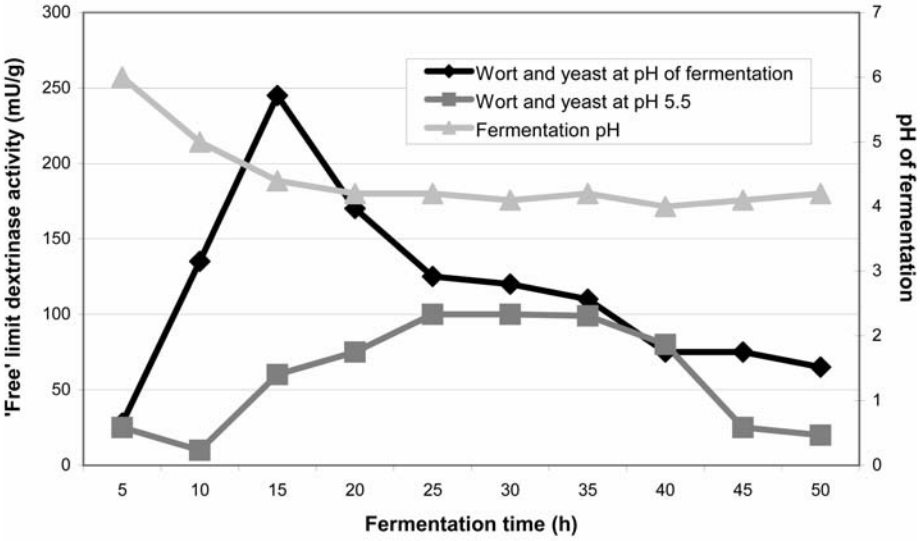

Fig. 1. The activity of 'free' limit dextrinase measured during 44 h fermentation. Limit dextrinase was assayed at the pH of the wort and after adjustment to pH 5.5. Gravity decreased from 1.060 to 0.998.

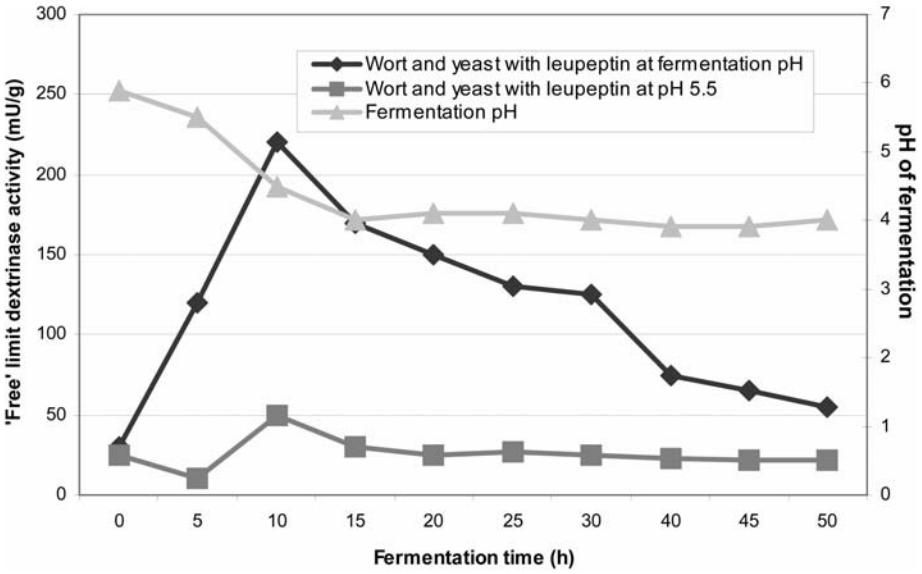

Fig. 2. The activity of 'free' limit dextrinase measured during a 44 h fermentation with added leupeptin. Limit dextrinase was assayed at the pH of the wort and after adjustment to pH 5.5. Gravity decreased from 1.060 to 0.998.

and total activity of 506 mU/g obtained from extractions at pH 5.5 without and with DTT. Initial limit dextrinase activity values in the fermentation of just below 45 mU/g were similar to malt free activity values implying the survival of the enzyme during mashing at 65°C.

The gravity decreased from 1.060 to 0.998 and 0.999 during 44 h of the control fermentation (Fig. 1) and fermentation with leupeptin (Fig. 2) respectively. The change in activity of limit dextrinase, during fermentation, measured at wort pH showed a very similar trend in the presence or absence of leupeptin. Activity of limit dextrinase in samples assayed at wort pH, increased to a maximum value of 240 mU/g following 8 h of fermentation (Fig. 2) and this activity increase was accompanied by a decrease in wort pH from 5.0 to 4.5. Assuming that leupeptin is active against cysteine proteases at low pH values, results

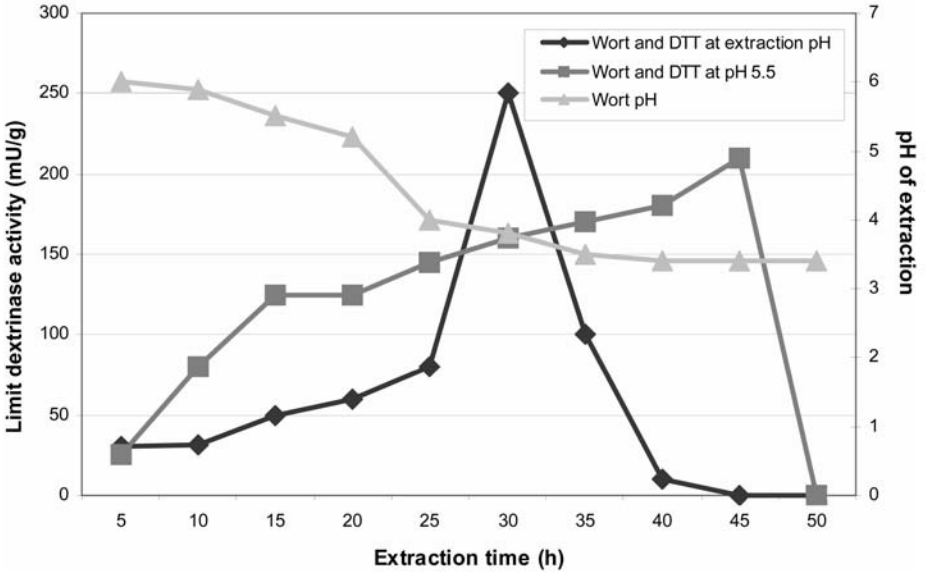

Fig. 3. The effect of 5 mM dithiothreitol (DTT) in wort on the extraction of limit dextrinase over 44 h. Limit dextrinase was assayed at the pH of the wort and at the adjusted pH of 5.5. Gravity decreased from 1.060 to 1.058.

lead to the conclusion that activation of limit dextrinase during fermentation is associated with the drop in pH and that limit dextrinase does not require cysteine proteases for this increase in activity.

Wort samples fermented with leupeptin and adjusted to pH 5.5 (Fig. 2) showed a lower limit dextrinase activity in comparison to those obtained during the control fermentation, activity was constantly low with a peak activity failing to reach over 50 mU/g in the presence of leupeptin. The increase in activity seen at pH 5.5 (Fig. 1) may thus reflect activation of limit dextrinase by cysteine proteases[37] surviving into the fermentation.

The effect of prolonged extraction of limit dextrinase from wort in the presence of DTT

Two serendipitous experiments were conducted in which the prolonged extraction of wort, after mashing at 65°C, was carried out in the presence of dithiothreitol (Figs. 3 and 4). The aim was to discover the extent to which this 'total' enzyme could be extracted following mashing and find out if 'total' activity would exceed 'free' activity found at reduced pH during fermentation (Figs. 1 and 2). In the first experiment, no yeast was added to the wort and there was no significant change in gravity over 50 h. Addition of DTT to the wort caused the pH to remain above pH 5.5 for the first 4 h (Fig. 3), this initial pH value was lower than that of the control fermentation pH (Fig. 1). The initial limit dextrinase activity (Fig. 3) was low, similar to the activity values obtained following mashing at 65°C (Figs. 1 and 2). These initial activity values, despite being low, further confirm the ability of the enzyme to survive into fermentation. Between 15 and 20 h of extraction, the wort pH dropped from 5.5 to 4 and the limit dextrinase activity increased dramatically to a maximum value of 240 mU/g (Fig. 3). This drop in pH was almost certainly due to bacterial growth and metabolism in the unboiled wort. After 20 h of extraction, the wort pH had dropped to 3.5 and the limit dextrinase activity decreased to a negligible value at 35 h. Limit dextrinase measured in wort at a buffered pH of 5.5 showed an increase in activity from a value of ca. 25 mU/g soon after mashing (5 h, Fig. 3) to ca. 225 mU/g following 44 h of extraction. Thus in the presence of dithiothreitol, higher activity of limit dextrinase (ca. 200 mU/g) was found at pH 5.5 compared to ca. 100 mU/g without DTT (Fig. 1). By 50 h of extraction, the limit dextrinase activity of the wort, measured at pH 5.5, disappeared and was equivalent to the activity assayed at wort pH, perhaps due to proteolytic degradation.

In a second experiment, yeast was added to the extraction with DTT in order to produce a mimic of fermentation. The gravity decreased from 1.060 to 1.049 during 50 h. The absence of the rapid pH drop seen in Fig. 3 (20 to 25 h) suggests that the yeast growth, although clearly minimal, could to some extent have reduced microbial growth but the pH did drop steadily throughout the first 30 h of extraction. When assayed at wort pH, the limit dextrinase activity reached a maximum value of ca. 250 mU/g after 24 h by which time the pH had declined from ca. 5.57 to ca. 3.8. When compared to activity values of the control fermentation (Fig. 1), this peak activity was similar and occurred at a lower pH. Buffered wort showed a peak activity of ca. 170 mU/g following ca. 35 h of extraction, 11 h later and ca. 80 mU/g lower then that of samples at actual wort pH. These results show that after a mash at 65°C, limit dextrinase was not extracted at wort pH (initially close to pH 5.5), even in the presence of dithiothreitol, to give activities equivalent to those obtained by extracting from malt at pH 5.5. Also, while activities measured at pH 5.5 with dithiothreitol (Figs. 3 and 4) were

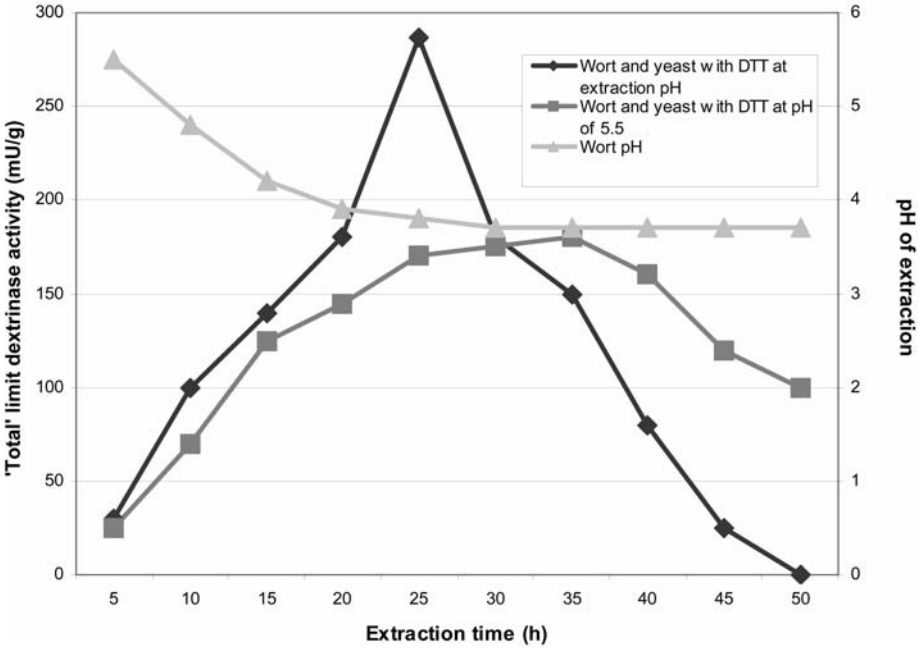

Fig. 4. The effect of 5 mM dithiothreitol (DTT) in wort on the extraction of limit dextrinase in the presence of yeast. Limit dextrinase was assayed at the pH of the wort and at adjusted pH of 5.5. Gravity decreased from 1.060 to 1.049.

slightly greater than in its absence (Figs. 1 and 2), the values were still below the maximum levels found when measured at wort pHs significantly lower than pH 5.5.

The effect of pH and extraction on 'free' and 'total' measurable limit dextrinase activity of malt

The data in Figs. 1 to 4 support the hypothesis that mashing temperatures reduce the potential to extract 'total' limit dextrinase from wort, but suggest that a reduced wort pH leads to a measurable activity higher than free activity measured at pH 5.5. Limit dextrinase of aerobic and anaerobic malt was extracted for a rapid 24 min and the conventional 5 h (Fig. 5). The 24 min is essentially as quickly as the extraction can be carried out and the extract prepared for assay. Limit dextrinase activity at pH 5.5, following rapid and conventional extraction, was low at values ca. 50 mU/g, on reducing this pH to 4.4 an increase of ca. 150 mU/g was observed (Fig. 5). The aerobic malt, extracted at pH 5.5 in the presence of DTT showed a similarly low initial activity of ca. 50 mU/g following 24 min extraction. Conventional extraction at pH 5.5, in the presence of DTT, revealed an entirely different pattern, giving a total limit dextrinase activity of ca. 500 mU/g.

Anaerobically produced malt is known to have a high 'free' activity equivalent to activities found following extraction of 5 d aerobic malt with DTT[25]. Limit dextrinase activities of the anaerobically produced malt extracted in the presence of DTT indicated that the almost all of limit dextrinase was present in the free form (results not shown). When extracted at pH 5.5 for 24 min, anaerobic malt showed a limit dextrinase activity of ca. 400 mU/g after rapid extraction (Fig. 5). Following 5 h extraction of the anaerobic malt at pH 5.5, a limit dextrinase activity of ca. 500 mU/g was measured, this value was similar to that obtained in the aerobic malt after 5 h extraction at pH 5.5 in the presence of DTT. Anaerobic malt extracted at the lower pH of 4.4 had an initial activity of ca. 350 mU/g, but after 5 h extraction this activity had decreased to a value ca. 40 mU/g (Fig. 5).

These investigations of limit dextrinase activity following its extraction in mashing (Figs. 1–4) or after extraction at 40°C at different pHs and times (Fig. 5) suggest that the enzyme is rapidly extracted at pH 4.4 to a higher activity than obtained after 5 h at pH 5.5, but to a lower level than the total activity measured following extraction at 40°C for 5 h with DTT. A more detailed investigation of the pH effect was therefore carried out. 'Free' and 'total' limit dextrinase activity of malts were determined after extraction for 0.5 h and 5 h at 40°C at pHs ranging from 4.0–5.5 (Figs. 6 and 7). A temperature of 40°C was chosen because this is the recommended temperature at which the malt extractions and assays are carried out[26]. After 0.5 h of extraction at pH 4.0, a 'free' activity of ca. 100 mU/g was obtained (Fig. 6), this 'free' activity had a maximum value of 190 mU/g after extracting and assaying at pH 4.5. At pHs greater than 4.5, after 0.5 h extraction, there was a decrease of the 'free' activity to a minimal value of 40 mU/g occurring at pH 5.5.

'Free' limit dextrinase activity was negligible at pH 4 following 5 h extraction, as the pH used for extraction

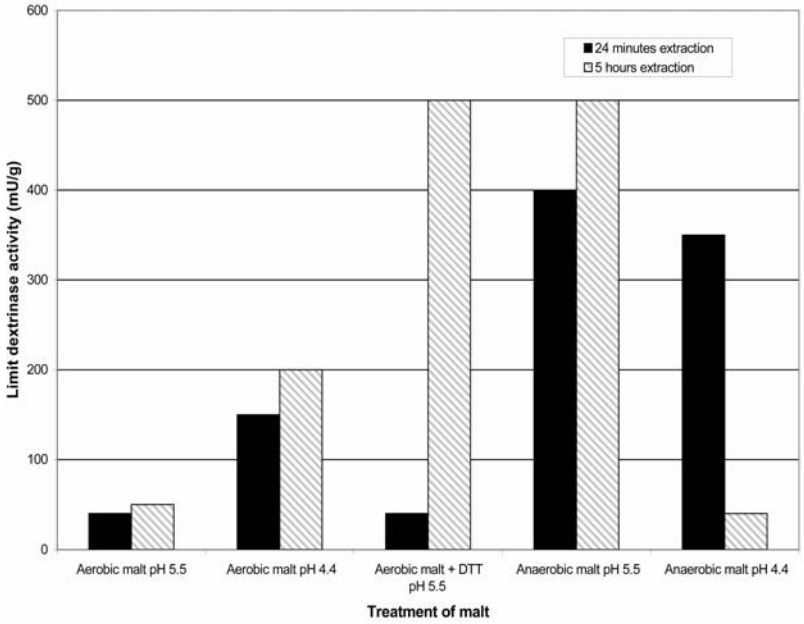

Fig. 5. The effect of extraction time, pH and 5 mM dithiothreitol (DTT) on the measurable activity of limit dextrinase from malt, extracted at 40°C. The aerobic malt was micromalted for 5 d and the anaerobic malt was micromalted for 5 d aerobically and 6 d anaerobically (see materials and methods for experimental details).

was increased to 4.25 and 4.5 the 'free' activity was ca. 110 mU/g. Peak 'free' activity was observed at pH 4.75 following 5 h extraction, increasing the pH above 4.75 caused a decrease in 'free' activity which was at its lowest at a pH of 5.25 (Fig. 6). Increasing the pH from 5.25 to 5.5 led to a minimal 10 mU/g increase in 'free' activity.

After 5 h extraction at pH 4, all limit dextrinase activity was lost. At extraction pH 4.25, the limit dextrinase activity showed a decrease of 40 mU/g between 0.5 h and 5 h extraction. A decrease of 10 mU/g occurred between 0.5 h and 5 h extraction at pH 4.75. There was little change in limit dextrinase activity at pH 5 during the additional 4.5 h of extraction, it remained stable. A 10 mU/g decrease was found to take place during conventional 5 h extraction at pH 5.25. At pH 5.5, the limit dextrinase activity was essentially unchanged over the 5 h extraction. Comparing 'free' limit dextrinase activities of rapid and conventional extraction leads to the conclusion that 'free' activity is highest following 0.5 h extraction at an optimum pH of around 4.5. After the conventional 5 h extraction, it can be proposed that limit dextrinase has been degraded by proteases which have optimum activity at the lower pHs around 4.5[37], with no limit dextrinase activity thus observed at pH 4.0. It appears from these results that the optimum pH for 'free' limit dextrinase activity is close to 4.5.

Fig. 7 shows that the results of extraction carried out with DTT present for 0.5 h or 5 h (conventional extraction time for total enzyme). A different pattern emerged compared to Fig. 6, particularly after 5 h. After 0.5 h extraction at low pHs of 4 and 4.25 the 'total' limit dextrinase activity remained below 30 mU/g, the lowest 'total' activity occurred at pH 4. As the pH was increased the 'total' activity increased over the 0.5 h extraction, with a peak activity of ca. 380 mU/g occurring at pH 5. When the pH was increased further to pH 5.25 and 5.5 the 'total' limit dextrinase activity decreased to a value of ca. 100 mU/g at pH 5.5.

After 0.5 h extraction at pH 4.5, the total limit dextrinase activity was ca. 130 mU/g (Fig. 7), which compared to a higher 'free' activity of ca. 190 mU/g (Fig. 6). After 5 h of extraction this activity had decreased to ca. 100 mU/g (no DTT, Fig. 6). After 0.5 h extraction at pH 4.75 the 'total' activity had increased to a value just over 300 mU/g, this activity had disappeared by 5 h. The highest 'total' activity, after 0.5 h of extraction, was observed at a pH of 5 (380 mU/g), by 5 h this activity had decreased to 100 mU/g. As the pH was increased to 5.25 and 5.5, the activity decreased to 150 and 100 mU/g respectively after the 0.5 h extraction. Interestingly, the 'total' activity decreases after 5 h at pHs 4.0–5.0 contrasted with a 550 to 600 mU/g increase in activity at pHs 5.25 and 5.5 after 5 h of extraction. This high 'total' activity at pH 5.25 and 5.5 after 5 h extraction contrasted with low 'free' limit dextrinase activity at these pHs. At pHs below 5, activity extracted after 0.5 h was to a large extent lost.

Effect of wort pH on limit dextrinase activity during prolonged incubation/fermentation

The data in Figs. 5 to 7 have established that limit dextrinase from malt extracts had an activity without activation by a reducing agent which is optimal at ca. pH 4.5,

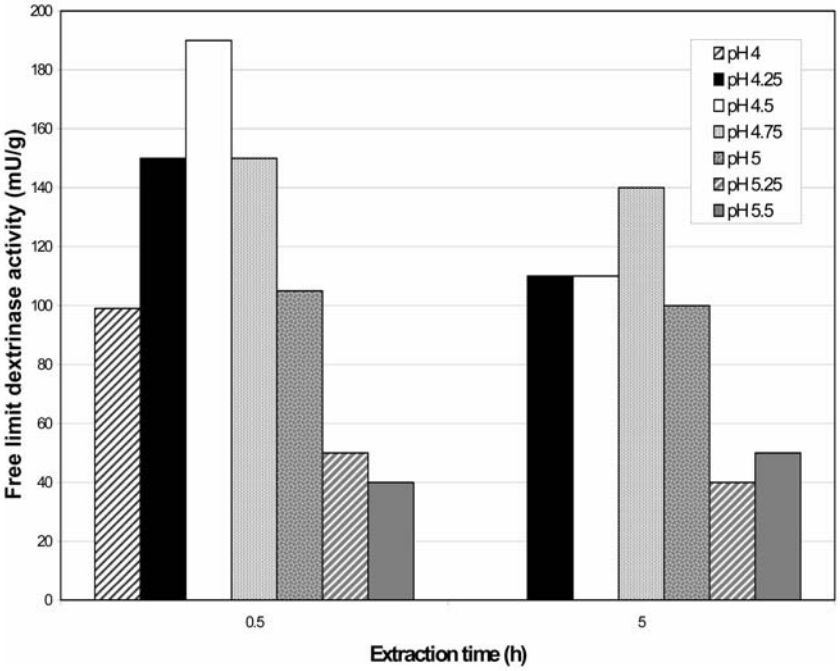

Fig. 6. The effect of pH on extraction (after 0.5 h and 5 h) and assay at 40°C of limit dextrinase in aerobically germinated malt in the absence of DTT.

rather than pH 5.5. This fits with the hypothesis that the activation of limit dextrinase seen in wort at low pHs (whether the pH drop is due to yeast fermentation or microbial infection) is the result of the decline in pH to ca. 4.5 or below and does not involve reducing conditions or the potential activation by cysteine proteases. To confirm this, the activity of five worts was monitored for limit dextrinase at 3 time points during 44 h, immediately after wort production (time 0 h), at 7 h and at 44 h after wort production (Fig. 8A). The pH of the wort at each time of assay was also determined (Fig. 8B). In the experiment, wort was incubated at its normal pH, wort was incubated with its pH adjusted to 4.4 at the start, wort with yeast was fermented at normal pH and wort with yeast was fermented with the pH adjusted to 4.4 at the start of fermentation with and without leupeptin, the cysteine protease inhibitor.

Limit dextrinase activity of the wort without yeast stayed low at a value below 20 mU/g for 44 h (Fig. 8A). When the pH of the wort was adjusted to 4.4 prior to incubation, it showed initial activity of ca. 130 mU/g, following 7 h it increased by 20 mU/g to a value of 150 mU/g. After 44 h, however, no activity was found. The protease inhibitor leupeptin was added to the wort and the pH adjusted to 4.4. The activities obtained were almost identical to activities in the absence of leupeptin (at time 0 h the activity was ca. 130 mU/g, at 7 h it was ca. 150 mU/g and at 44 h it was 0 mU/g) showing that cysteine proteases are probably not responsible for this lack of activity at 44 h. Fig. 8B shows the pH of a wort without yeast, decreased from 4.4 to ca. 3 and similarly wort with leupeptin dropped to a pH ca. 2.5, these pHs are both almost certainly too low for any significant limit dextrinase activity[6].

Yeast was added to wort, and limit dextrinase assays were carried out at wort pH. Activity was initially low, lying just below 20 mU/g, this increased dramatically by 7 h to a value just over 180 mU/g, by 44 h the activity had decreased to ca. 130 mU/g. When the fermentation pH was adjusted to 4.4, the initial limit dextrinase activity was just over 120 mU/g, this value can be compared to the activity of ca. 18 mU/g obtained at normal fermentation pH. This activity of 120 mU/g remained stable for the first 7 h of fermentation but, by 44 h fermentation the activity was negligible.

Fig. 8B showed the fermentation pH to be initially ca. 5.5, at this pH the activity was ca. 18 mU/g (Fig. 8A). After 7 h, the pH decreased to ca. 4.3, this change in pH was accompanied by an increase in limit dextrinase activity to ca. 180 mU/g. At 44 h the pH decreased further from 4.3 to 4.1, and the limit dextrinase activity had decreased further to a value of ca. 130 mU/g. When fermentation pH was altered from normal wort pH (ca. 5.5) to 4.4 the limit dextrinase activity increased from a value of ca. 18 mU/g to ca. 120 mU/g. The pH of the fermentation with starting pH 4.4, decreased to pH ca. 3.5 after the first 7 h and these pH values were accompanied by an activity of ca. 123 mU/g when the pH decreased further to a value of 3.4 following 44 h fermentation. There was no measurable limit dextrinase activity at this time. Wort with the initial pH adjusted to 4.4 displayed a pH decrease of ca. 1 during the first 7 hours of fermentation with yeast yet

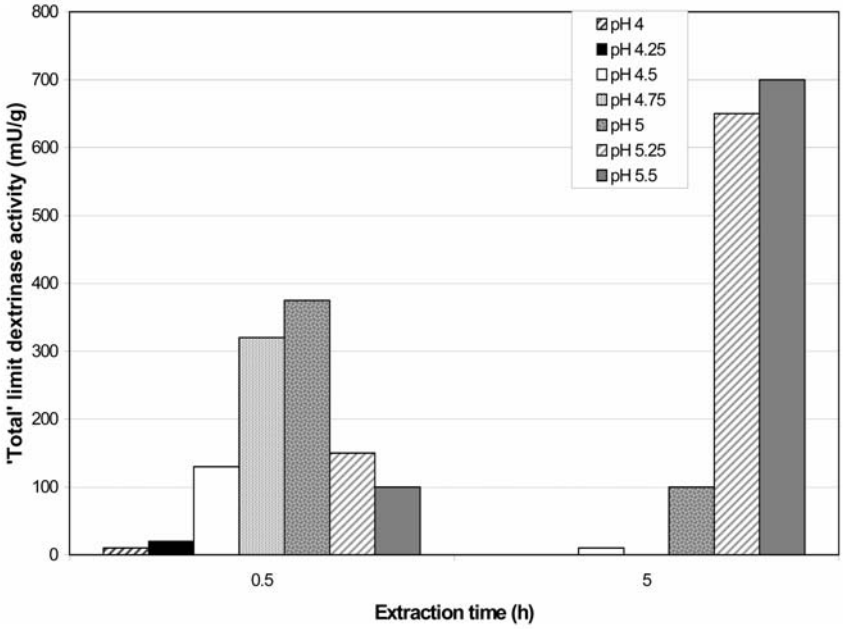

Fig. 7. The effect of pH on the extraction (after 0.5 h and 5 h) and assay at 40°C of 'total' limit dextrinase in aerobically germinated malt in the presence of 5 mM DTT.

despite this decrease the limit dextrinase activity remained stable, the pH then decreased further by 0.1 and this minute change resulted in negligible activity 36 h later.

In the previous experiments with wort, pH adjustment occurred following mashing. The effect of altering the mashing pH on limit dextrinase activity was investigated. Samples were mashed for 1 h at pHs from 4.46 to 5.76, cooled and assayed at 40°C for limit dextrinase activity at the particular pH of the mash or after adjustment to pH 4.4 using HCl (Fig. 9). A substantial increase in limit dextrinase activity was only found on reduction of the pH if it was adjusted to 4.4 with the mashes at pH 5.66 or 5.76. Mashing at pH 5.37 failed to extract the enzyme or led to processes during mashing which inactivated it. Following a 1 h mash, initial limit dextrinase activities at pH 4.46, 4.77 and 5.14 were all low at values below 20 mU/g. A mash of pH 5.37 had the highest limit dextrinase activity at a value of ca. 27 mU/g. Adjusting the pH to 4.4 prior to assaying generally increased the limit dextrinase activity, the most clear cut examples of increased activity as already stated included those of pH 5.66 and 5.76, with activities of 107 and 124 mU/g respectively. These results suggest that the enzyme is protected from denaturation at mashing temperatures when in complex with its proteinaceous inhibitor.

DISCUSSION

Several experiments revealed that the optimum pH for limit dextrinase assays in the presence of inhibitor lie between 4.2 and 4.5, not the pH of 5.5 recommended by the assay from Megazyme[26]. The increase in 'free' limit dextrinase activity at a pH range of 4.2 to 4.5 was observed in the standard fermentation with and without leupeptin (Figs. 1 and 2), the 44 h extraction in the presence of DTT (Fig. 3) and extraction with DTT and yeast (Fig. 4).

During the standard fermentation (Fig. 1) increases in limit dextrinase activity were associated with decreased pH. At wort pH 4.4, the limit dextrinase rise reached a peak of 250 mU/g, a value that is lower than the optimum obtained from assay of malt following 5 h extraction in the presence of DTT. During fermentation, at wort pH, slow degradation of the limit dextrinase inhibitor protein by proteases could be evident over ca. 20 h extraction. This conclusion is based on the finding that buffering the wort to pH 5.5 resulted in reduced limit dextrinase activity, but this reduction declined over the period of extraction, suggesting that less inhibitor was available to inhibit limit dextrinase at pH 5.5. Following maximum 'free' activity, at wort pH 4.4, the pH declined further reducing the limit dextrinase activity. The wort pH then appeared to stabilise at ca. 4, the limit dextrinase activity however continued to decline possibly due to steady proteolytic breakdown.

As just stated, assay at the buffered pH of 5.5 showed an increase in limit dextrinase up to 20 h in the absence of protease inhibitor but not in its presence. This indicates regulation of limit dextrinase activity by cysteine proteases which may have exerted an effect by degrading the inhibitor, thus activating the limit dextrinase. Continued proteolytic and other denaturing effects after 30 h probably account for the slow reduction of limit dextrinase seen in the assays at both wort pH and pH 5.5.

The presence of leupeptin during fermentation (Fig. 2) did little to alter the limit dextrinase activity at the pH of the wort, similar trends in the fermentation and limit dex-

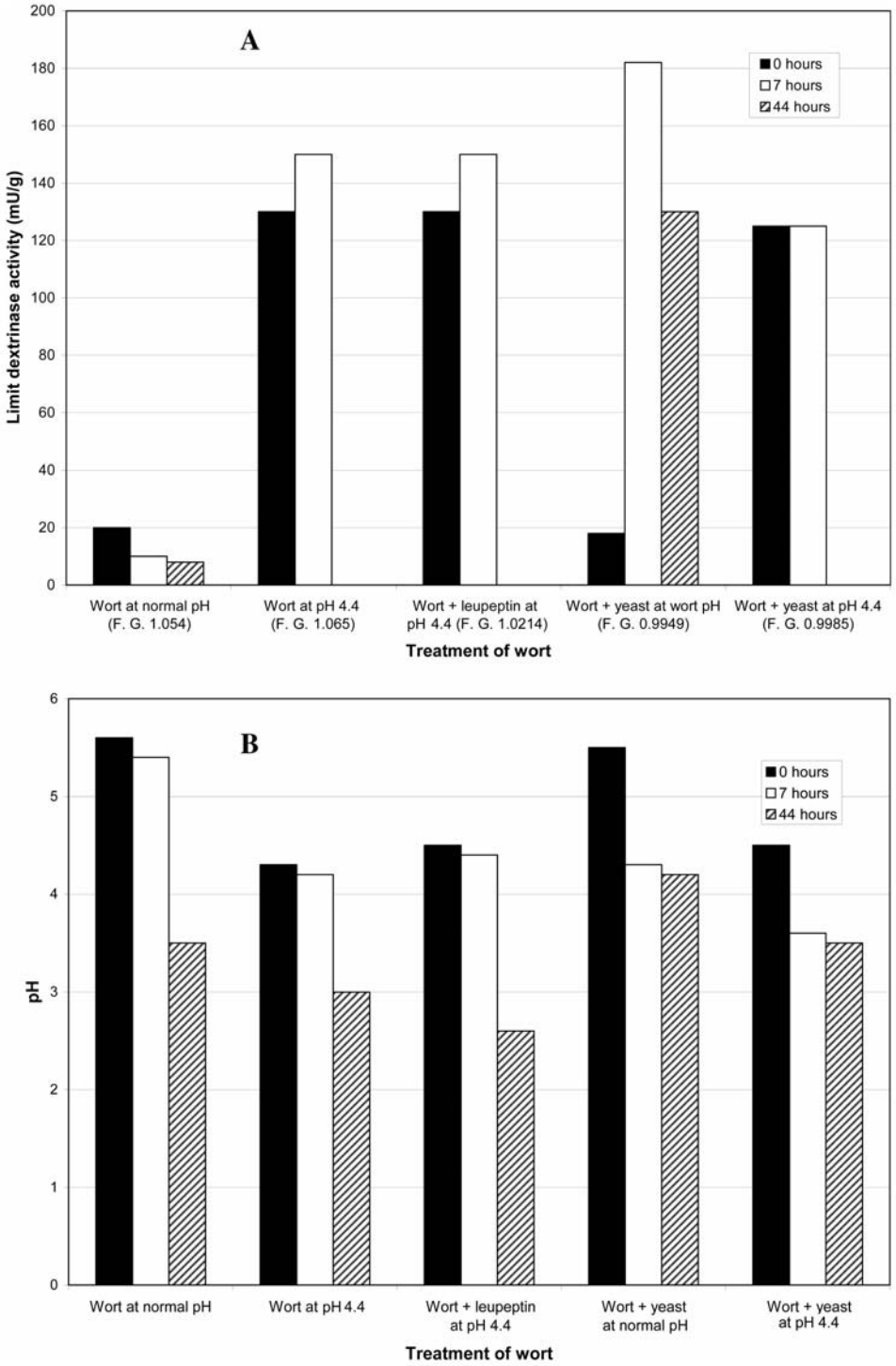

Fig. 8. A, 'Free' limit dextrinase activity of wort at normal wort pH, or with wort pH adjusted to 4.4 at the start of incubation/fermentation, during 44 hours of incubation/fermentation. Where no data appears to be shown, the activity was zero. F.G. shows final specific gravity. **B,** The change of wort pH during 44 h incubation/fermentation. The starting pH was either unadjusted (normal) or adjusted to 4.4 at the start of incubation/fermentation.

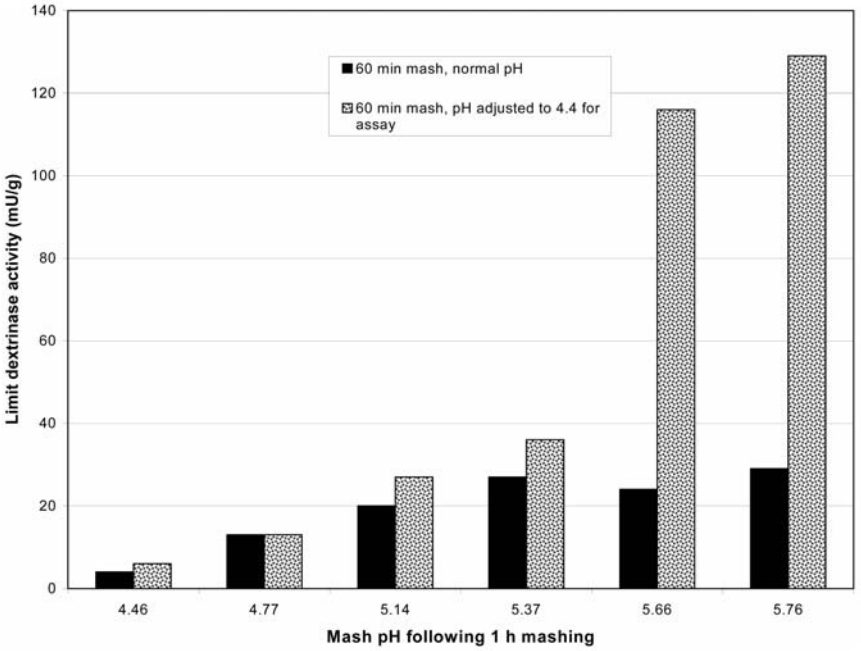

Fig. 9. The effect of mash pH on the 'free' limit dextrinase activity following 1 h mashing at 65°C at mash pH or after adjustment to pH 4.4 for assay.

trinase activity were observed. An increase in limit dextrinase was seen in the first 8 h of fermentation, reaching maximum activity at a pH ca. 4.5. This was however substantially lower then the optimum obtained for the assay of the malt following 5 h extraction with DTT[15]. The presence of leupeptin in the fermentation probably ensured a lack of inhibitor degradation by cysteine proteases, therefore the pH decrease could be responsible for the limit dextrinase peak activity and low pH must alleviate the effectiveness of the limit dextrinase inhibitor. When fermentation pH was buffered to 5.5, the inhibitor protein could have re-associated with the limit dextrinase, this resulted in a continuously low limit dextrinase activity. Cysteine proteases must be important for the activation of limit dextrinase at pH 5.5 but are not required for limit dextrinase activation at lower pHs. The inhibitor may become inactive at lower pHs[28]. Cysteine proteases have however been shown to be more active at a pH range of 3.4 to 4.5[37–39] and the lag before an increase in activity at pH 5.5 (Fig. 1) could be due to a relatively low cysteine protease activity at this pH.

DTT was added to extractions to discover the extent of which total enzyme could be converted to active free enzyme following mashing (Figs. 3 and 4). The pH adjusted assays containing DTT produced trends similar to these assays from the fermentations (Figs. 1 and 2), but DTT achieved a higher limit dextrinase activity at the adjusted pH of 5.5. The presence of yeast (Fig. 4 compared to Fig. 3) had little effect on the activity of limit dextrinase measured at pH 5.5, probably because there was almost no fermentation in the presence of DTT. In extractions containing DTT (Fig. 3), a drop in wort pH occurred after 15 h of extraction, which was attributed to contamination of the unsterilised wort by lactic acid bacteria. The pH changes probably correspond with lactic acid bacterial metabolism[22]. This sudden drop in pH was accompanied by an increase in limit dextrinase activity. Following peaks in limit dextrinase activities in the presence of DTT at wort pH (Figs. 3 and 4), the limit dextrinase activity decreased dramatically. The decrease in activity could be attributed to proteolytic degradation of limit dextrinase but it is probable that mashing temperatures would have significantly reduced the activity of proteases. At the adjusted pH of 5.5, limit dextrinase activity continued to increase up until 38 h fermentation and after peak activity at this adjusted pH of 5.5 the activity remained relatively stable while the activity of limit dextrinase at the wort pH dropped radically. It is most likely that this decrease of activity was due to the low pH of the wort. The assays at pH 5.5 establish that the enzyme was present and therefore the decreasing activity measured at 35 h and 40 h (Figs. 3 and 4), must have been due to the low pH of the wort.

The effects of extraction time, pH and DTT on measurable malt limit dextrinase activity, extracted at 40°C, were investigated in aerobic and anaerobic malts (Fig. 5) to establish what effect pH would have on measurable limit dextrinase in the absence of extraction at a mashing temperature of 65°C. Aerobic malt extracted at pH 5.5 showed an initially low activity which did not appear to increase after 5 h of extraction, this suggests low activity of cysteine proteases and the lack of reducing conditions have failed to activate the enzyme, this is typical of malt analyses. Aerobic malt extracted at pH 4.4 displayed a higher limit dextrinase activity than that of the aerobic

malt at pH 5.5, this higher activity was present following rapid extraction. The 5 h extraction of aerobic malt at pH 4.4 produced only marginally higher 'free' limit dextrinase activity. This suggests stability of the enzyme at this pH. Anaerobic malt at pH 5.5 showed the greatest free limit dextrinase activity following both rapid and conventional extraction, a value similar to the total limit dextrinase activity obtained for aerobic malt at pH 5.5.

It has been well documented that the onset of anoxia is associated with a decrease in cytoplasmic pH in plants[12,29]. Several parameters are involved in the regulation of cytoplasmic pH in anoxic plant cells. The increase of cytoplasmic H^+ ions can be attributed to the passive influx of H^+ ions from the vacuole or external environment, the hydrolysis of pools of Mg nucleoside triphosphate (NTP) and sugar phosphates[1], the accumulation of non-processed acidic intermediates[8], the synthesis of lactate and poor CO_2 removal. The decrease in pH and the synthesis of lactate in anoxic tissues suggests that lactate accumulation is the most likely cause of cytoplasmic acidification[30], but anoxia can also cause acidification of the cytoplasm in tissues where lactate synthesis is low. In anoxic sycamore (*Acer pseudopantanus*) cells the release of H^+ ions accompanying the Pi liberating hydrolysis of NTP was the principle cause of cytoplasmic pH drop and the H^+ pumping ATPase located in the plasma membrane was also blocked[10]. In the barley endosperm, acidification occurs at the late stages of grain germination and is caused by the accumulation of malic acid[22,27], at this stage of grain germination the activity of debranching enzymes such as limit dextrinase would be of utmost importance to degrade dextrins produced following starch hydrolysis by amylases.

Anaerobic malt, extracted and assayed at pH 4.4 displayed activities almost as high as achieved at pH 5.5 after 24 min extraction. Thus in anaerobic malt, proteases probably degraded the inhibitor so that on adjustment to pH 5.5 there was no significant binding of inhibitor and a fall in limit dextrinase activity. The higher activity of limit dextrinase after 24 min extraction at pH 5.5 compared to pH 4.4 (Fig. 5) shows that the activity measured at 4.4 is suboptimal compared to that obtained at pH 5.5, but it is interesting that the activity of aerobic malt limit dextrinase at pH 4.4 was less than 50% of that at pH 5.5 extracted for 5 h with DTT, whereas the anaerobic malt activity at pH 4.4 was quite close to that found after 5 h at pH 5.5. This suggests that even at pH 4.4 activities of limit dextrinase are reduced by the inhibitor.

A more comprehensive examination of the effect of pH on extraction and assay of limit dextrinase in the presence and absence of DTT showed that in the first 0.5 h (Figs. 6 and 7) the highest 'total' activity with DTT occurred at pH 5 (Fig. 7) and peak 'free' activity without DTT was at pH 4.5 (Fig. 6). Conventional 5 h extractions gave a reduced 'free' activity at pH 4 to 4.5 (Fig. 6) this is probably due to protease activity at these pHs. Conventional 5 h extractions also showed 'total' limit dextrinase activity to be at its peak at pH 5.5 as is normally found, this high 'total' activity was coupled to a low 'free' limit dextrinase activity (Fig. 6). The results shown in Figs. 6 and 7 suggest that a pH range of 5.25 to 5.5 to be optimal for limit dextrinase inhibitor binding. 'Free' limit dextrinase activity after 5 h extraction at pH 5.5 (Fig. 6) produced low values, which correspond with values attained during 5 h extraction of aerobic malt at pH 5.5 (Fig. 5). 'Total' limit dextrinase extraction (Fig. 7) is consistent with a slow cysteine protease activity at pHs 5.25 and 5.5. The suboptimal activity, which is found at pH 4.4 is shown by comparing the 'free' activity, ca. 140–190 (Fig. 6) to the 'total' activity ranging from 400 to 500 mU/g (Fig. 7). It is startling that in the presence of DTT (Fig. 7) the activities at pH 5 and below were significantly lower than in the absence of DTT. This is probably accounted for by high activities of proteases at these lower pHs in the presence of DTT that degrade limit dextrinase. The enzyme in the experiments presented in Figs. 6 and 7 had not been subjected to mashing temperatures, which could have denatured proteases.

The limit dextrinase activity of wort at pH ca. 5.6 (pH of wort unaltered), following mashing was low (Figs. 8A and 8B). Wort incubated at the lower pH of 4.4 showed a 550% increase in initial limit dextrinase activity following mashing, after 7 h the pH decreased very slightly and the limit dextrinase activity increased by 20 mU/g. After 44 h the pH was 3 (Fig. 8B) and the activity was non-existent (Fig. 8A). This pH 3 is below the pH for measurable limit dextrinase.

To establish the level of involvement of cysteine proteases in this pattern, leupeptin was added to the extract prior to incubation. Wort incubated at pH 4.4 in the presence and absence of the cysteine protease inhibitor produced similar results to those found in its absence. This adds further weight to the conclusion that cysteine proteases, despite being enzyme activators, are not required for enzyme activation at pH 4.4, this conclusion is based on the assumption that leupeptin will act as an inhibitor at pH 4.4. These results support the hypothesis that at pH 4.4 the enzyme dissociates from the inhibitor. However, 'free' limit dextrinase is more vulnerable than its bound counterpart and easily denatured by increasing temperature[6] and decreased pH (Fig. 9). Reduction of wort pH is responsible for an increase in limit dextrinase activity. Evidence shows that replacing 40% of cook water with backset causes a drop in pH from ca. 6.2 to ca. 4.4, this pH decrease results in a limit dextrinase activity nine times higher then in normal control mashes. This increased limit dextrinase activity was associated with decreased amounts of branched dextrins in the wort and thus increased fermentability (C. S. Cooper, personal communication).

Maximum 'free' limit dextrinase activity, ranging from 150 to 190 mU/g, has been shown to occur at pHs 4.25, 4.5 and 4.75 following rapid extraction (Fig. 6). At low pHs of 4.46 and 4.77, following 1 h mashing, the 'free' limit dextrinase activity was low (Fig. 9), this was however not unexpected because the majority of the limit dextrinase was in its unprotected 'free' form and thus denatured during 1 h mashing (65°C). Maximum 'total' activity was observed at pHs of 5.25 and 5.5 following 5 h extraction (Fig. 7). 'Free' activity was lost following 1 h mashing at pHs 5.14 and 5.37 (Fig. 9), thus the majority of the enzyme must be considered to be in the unbound form at these pHs. At the higher pHs of 5.66 and 5.76 for mashing, most of the limit dextrinase will be in bound form. There is evidence that the inhibitor when in complex with

the enzyme provides protection against mashing temperatures allowing the enzyme to survive into fermentation[6]. When the pH is adjusted to less than 5.4 (Fig. 9) and the inhibitor presumably starts to dissociate from the limit dextrinase, it becomes sensitive to temperature.

In summary, limit dextrinase can be activated by DTT and cysteine proteases[15]. There is evidence that the enzyme-inhibitor complex dissociates at pHs below 5 increasing the 'free' limit dextrinase activity in wort but at pH 5.5, the optimum pH for limit dextrinase activity[26], the activity of the inhibited enzyme remains low. These results contrast with those obtained by Stenholm and Home[34] adding weight to evidence that malt limit dextrinase, extracted in the presence of its inhibitor[23], acts in a different way to the purified enzyme. Proteases from the malt, which have a pH optimum for activity ranging from 3 to 4[38,39] could degrade limit dextrinase at these lower pHs, but they are sensitive to high mashing temperature so their activity will be reduced significantly. However, the activity of yeast and bacterial proteases produced during fermentation could reduce the activity of limit dextrinase or indeed other hydrolytic enzymes. Thus if limit dextrinase is extracted at mashing temperatures of 65°C, then limit dextrinase has low activity. Anoxic germination reduces endosperm pH and the associated pH decrease could be responsible for increased limit dextrinase activity through a decrease in the level of binding of inhibitor protein. Limit dextrinase appears to be protected from proteolytic degradation in its inhibited form, at lower mashing pHs and temperatures proteases are more active[37–39], if limit dextrinase is not protected by its proteinaceous inhibitor it may well be degraded.

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The regulation of limit dextrinase activity in malting, mashing and fermentation

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Introduction

Limit dextrinase (EC 3.2.1.142) hydrolyses α-1,6 glucosidic linkages in pullulan, amylopectin, and branched dextrins derived from amylopectin (Bryce,2003; MacGregor, 2003). The renaissance of research on limit dextrinase began in the early 1990s following the development of assays based on hydrolysis of dyed pullulan (McCleary, 1992). Pullulan is a polysaccharide consisting of maltotriose molecules linked by α-1,6 glucosidic bonds. The hydrolysis of dyed pullulan by limit dextrinase releases lower molecular weight fragments from the pullulan chain. These dyed fragments are soluble and can thus be determined spectrophotometrically with a release of solubilised dye being correlated with limit dextrinase activity. In the procedures for measurement of malt limit dextrinase, the enzyme can either be extracted at pH 5.0-5.5 for 5 hours at 40°C with a reducing agent to obtain the 'total' enzyme's activity or without a reducing agent to obtain the 'free' enzyme (soluble and without activation due to a reducing agent) (McCleary, 1992).

In the late 1980s, malt limit dextrinase was regarded as an enzyme with low activity that was heat-sensitive and therefore it was thought that to a very large extent limit dextrinase would be inactivated by kilning, and that any surviving enzyme would be rapidly denatured during mashing.

The extensive research that has been done on limit dextrinase over the last 15 years portrays a very different scenario. It has been discovered that the enzyme can survive both kilning and mashing temperatures (Sissons, Taylor and Proudlove, 1995; MacGregor, Macri, Bazin and Sadler, 1995; Stenholm and Home, 1999; Walker, Bringhirst, Broadhead, Brosnan and Pearson, 2001; McCafferty, Jenkinson, Morris and Bryce, 2004). 'Total' limit dextrinase that can be extracted from malt (ie extraction with a reducing agent) differs between barley varieties and between malts. There is good evidence that reducing agents activate proteases which then activate limit dextrinase (Longstaff and Bryce,1993).

It is now known that in barley, there is a proteinaceous inhibitor of limit dextrinase and that this inhibitor is degraded during malting (Macri, MacGregor, Schroeder and Bazin, 1993, MacGregor, Macri, Schroeder and Bazin, 1994; MacGregor *et al.*, 1995; MacGregor, 2004; Stahl, Alexander, Coates, Bryce, Jenkinson and Morris, 2007). Barley varieties however differ in their level of inhibitor and the rate at which it is degraded (Ross, Sungurtas, Ducreux, Swanston,

Davies and McDougall, 2003). Transgenic barley has been produced using antisense technology to down-regulate inhibitor levels in barley grains. This barley has altered starch composition and has been used to investigate the function of the inhibitor (Stahl, Coates, Bryce and Morris, 2004). Furthermore, pH affects the binding of inhibitor to limit dextrinase in the mash and fermentation, and thus the potential of limit dextrinase to degrade α-1,6-glucosidic bonds (McCafferty *et al.*, 2004).

Malt enzymes are a major factor in determining the composition of wort. An increase in limit dextrinase activity during mashing and fermentation will allow greater breakdown of branched dextrins and enhance the fermentability of wort. Thus, increased limit dextrinase activity during the production of spirit will increase alcohol production with ensuing economic benefits to the distilling industries. This work was carried out with the aim of investigating the variation in limit dextrinase activity of individual grains from samples of malted barley.

Materials and methods

Chemicals and biological material

Barley *Hordeum vulgare* (variety Optic) was supplied by a local maltings. Chemicals were obtained from the Sigma Chemical Company, Poole, Dorset, England or BHD, Leicestershire, England and were of analytical or the highest grade available. Limit Dextrizyme Kits were from Megazyme International, Bray, Wicklow, Ireland.

Malt production

Normal aerobic germination was carried out using a Seeger micromalting plant (McCafferty *et al.*, 2004). Aerobic malt was germinated for 5 d or 6 d and then freeze dried prior to analysis.

Limit dextrinase extraction and assay

Limit dextrinase of malts was extracted and assayed in 100 mM sodium maleate buffer as specified by the Limit Dextrizyme method (McCleary, 1992). Ground malt was sealed in tubes and allowed to shake in an incubator at 40 °C for 5 h. The procedure was scaled down for individual grain analysis. 'Free' activity is the activity measured after extraction for 5 h without DTT, and 'total' activity is measured after 5 h extraction with DTT.

Results

Optic was malted in the Seegar micromalting plant and freeze dried after either 5 or 6 days of germination. Analysis was then carried out to determine the activity of limit dextrinase in 100 individual grains. Extraction and assays were at pH 5.5 for 5 h with and without DTT. Extraction with DTT provides a measurement of 'total' activity and without DTT a measurement of 'free' activity. Figure 1 shows the pattern of activities found in 100 individual grains after 5 days. The highest proportion of grains had activities of 2 mU/grain or less. Since the average weight of grains was 0.037 mg, this equates to an activity of 54 mU/g or less, an activity typical of 'free' limit dextrinase activities in malt. However, a number of grains showed activities up to 11 mU/grain or 297 mU/g. Overall, the distribution of 'free' activity in individual grains was heavily skewed to the lower end of the activity spectrum.

In contrast, the total activity of limit dextrinase, extracted and measured with DTT showed a spread of activities from 4 up to 16 mU/grain (Figure 1) or 108 to 432 mU/g. These higher activities would equate to levels of total activity found in malt (McCafferty *et al.*, 2004).

Figure 2 shows the activities found after a further day of germination (6 days). The pattern has changed. There are now 42% of grains with 'free' activities between 2 and 4 mU/grain or 54 and 108 mU/g. However, there is a general

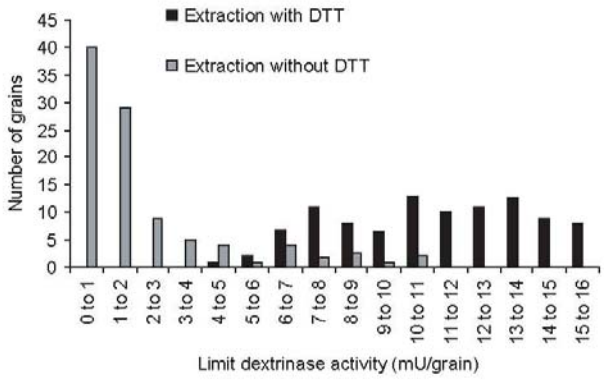

Figure 1. Limit dextrinase activity of 5 day aerobically germinated Optic grains (100).

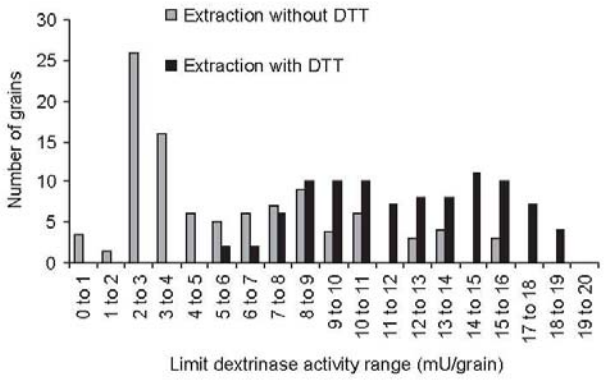

Figure 2. Limit dextrinase activity of 6 day aerobically germinated Optic grains (100).

spread in activities between 5 and 16 mU/grain or 135 and 432 mU/g. There was also an increase in the 'total' activities, with these increasing from 5 to 19 mU/grain on 135 to 514 mU/g.

Discussion

The activities of 'free' and 'total' limit dextrinase increased with an additional day of germination (Figures 1 and 2). This increase in free limit dextrinase activity of some grains led to a distinctly bimodal activity distribution pattern. An increase in activity of ß-glucanase from individual grain during germination was also seen by de Sá and Palmer (2004) who malted the varieties Decanter and Chariot.

Limit dextrinase has been found to increase as the pH is lowered from 5.5 to 4.4 and it has been hypothesised that this is due to unbinding of inhibitor from the enzymes (McCafferty *et al.*, 2004). The inhibitor binds at the active site of the enzyme with a stoichiometry of 1:1 (MacGregor, 2004). Therefore, the increasing activity of 'free' enzyme in individual grains over time is a reflection of two processes. These processes are the breakdown of inhibitor and the synthesis of new enzyme. The significantly higher activities of 'total' limit dextrinase compared to 'free'

enzyme of individual grains at 6 days (Figure 2) suggests that the 'free' activities measured are a reflection of both inhibited and uninhibited enzyme. However, whereas almost 70% of grains had activities of 0 to 2 mU/grain after 5 days, this was true for only 4% of grains at 6 days. The ability of normal malt to inhibit 'free' limit dextrinase of an anaerobic malt (McCafferty *et al.*, 2000), however, suggests that inhibitor may often in excess of that required to inhibit limit dextrinase. This means that at 5 days, many of those grains with low activity, probably contained inhibitor in excess of that required to inhibit their own limit dextrinase.

Cooper *et al.* (2003) showed that limit dextrinase activity increased during the fermentation of an unboiled all-malt mash, this increase was not seen where the mash pH was lowered by backset (Cooper *et al.*, 2003). It has since been shown that if the pH is lowered during the extraction of limit dextrinase, the limit dextrinase is more sensitive to inactivation by mashing temperatures (McCafferty *et al.*, 2004). Therefore, if a mash was at a pH close to 5.5, then the excess inhibitor from the malt of 5 days could have bound to 'free' enzyme during mashing and protected this enzyme from inactivation.

The activity of limit dextrinase during mashing and fermentation is thus a compromise between 'free' and inhibited enzyme. Inhibitor present during mashing will reduce the activity of limit dextrinase, but the protective effect of this inhibitor will allow enzyme to become active during fermentation as the pH declines. A knowledge of the activities of limit dextrinase present in individual grains will thus allow an understanding to be developed of limit dextrinase activities during mashing and fermentation in the distilling industries.

Acknowledgement

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The barley limit dextrinase inhibitor: Gene expression, protein location and interaction with 14-3-3 protein

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Abstract

The barley (*Hordeum vulgare* L.) limit dextrinase inhibitor (LDI) binds to and prevents the enzymic action of the starch debranching enzyme limit dextrinase (E.C. 3.2.1.142), and reduced levels of LDI have pronounced effects on starch biosynthesis including a loss of the small B-type starch granules. In this work, the time and place of *LDI* gene expression, and also the expression of LDI protein and inhibitory activity, and binding of LDI to the regulatory protein 14-3-3 were investigated. It was found that LDI is encoded for by a small multigene family, and the *LDI* gene is expressed at 2–4 weeks post anthesis in the endosperm. The LDI protein is present and active from 4 weeks post anthesis onward only in the starchy endosperm, not in the aleurone layer or other tissues, and is broken down during germination. Immunolocalisation shows the LDI protein to accumulate predominantly in the outer regions of the starchy endosperm, which coincides with the region where the small B-type granules are found. Additionally, the LDI protein sequence contains a 14-3-3 binding motif, and LDI binds to the barley 14-3-3A and 14-3-3C proteins in a phosphorylation-dependent manner, although addition of full length 14-3-3 proteins had no effect on LDI or LD activities *in vitro*. These findings are consistent with an important role for LDI (and by inference limit dextrinase) in the regulation of starch biosynthesis and starch granule formation.

Keywords: Barley; Limit dextrinase inhibitor; Limit dextrinase; 14-3-3

1. Introduction

Starch breakdown in the cereal endosperm during germination is accomplished by the concerted action of α-amylase and β-amylases, which cleave the α-1–4 linkages of the glucose polymer, and also by the starch debranching enzyme limit dextrinase (LD) (E.C. 3.2.1.142), which cleaves the α-1–6 linkages in branched amylopectin molecules (reviewed [1]). The action of LD, thought to be synthesised in the aleurone layer during germination and exported to the starchy endosperm, is critical for the supply of carbohydrates to the germinating cereal embryo, and is also of great importance in the malting industry for the production of fermentable sugars from barley grain (reviewed [2]).

When LD is extracted from germinating grains it is to be found at low levels as an insoluble bound form, higher levels of a soluble inactive form, and also the active free form; the inactive form can be activated by incubation with proteases or with reducing agents [3,4]. In its inactive soluble state, LD is thought to be combined with an inhibitor of LD, called the limit dextrinase inhibitor (LDI), which was first detected in barley by Macri et al. [5], and shown to consist of two low molecular weight proteins of differing isoelectric points but identical in sequence [6,7], and identical to the predicted protein product of a barley cDNA previously thought to be an α-amylase-trypsin inhibitor [8,9]. The *in vitro* complex formed between LDI and LD was found to consist of a 1:1 molar ratio of LDI to LD [10].

LDI inhibitory activity levels have previously been determined during barley germination, during which LDI activity drops off and negatively correlates with an increase in LD activity [11,12]. However, LD is also synthesised and active at low levels during grain development [13], and at this time may play a role in amylopectin synthesis. The coordinated activities of a number of different enzymes are required for

Abbreviations: LD, limit dextrinase; LDI, limit dextrinase inhibitor

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normal starch biosynthesis and granule formation, including starch synthases, starch branching enzymes and starch debranching enzymes [14]. The relative roles of the two classes of debranching enzyme, LD and isoamylase, is still a matter of some debate. In mutants that lack debranching activity, a highly branched form of starch, phytoglycogen, is formed, as is seen in the *sugary* mutants of maize [15] and rice [16]. Both the maize and rice *SU1* genes were found to encode isoamylase, however mutations in these genes have a pleiotropic phenotype, also conferring reduced LD activity [15,17–19]. The barley mutation *isa1* eliminates isoamylase activity, leading to phytoglycogen accumulation and compound starch granules, although levels of LD activity in this mutant are not clear [20]. A mutation in the maize LD (*zpu1*) gene has been described in which normal starch accumulates in the endosperm, however the *zpu1/su1* double mutant showed reduced starch levels compared to *su1* alone, suggesting a compensatory role of LD in the *su1* mutant [21].

We have recently shown that down-regulation of *LDI* expression by antisense had a profound effect on barley starch synthesis, including enhanced amylose to amylopectin ratios, changes to amylopectin architecture and much reduced levels of the small B-type starch granules [22]. Thus, LDI, probably, through an interaction with LD, plays an important role in starch synthesis and the development of the barley starchy endosperm. As yet, little is known about the dynamics of LDI gene expression or protein localisation. In order to enhance our understanding of how LDI might be involved in endosperm development, the time and place of *LDI* gene expression and LDI protein location in the barley grain was determined in the work presented here.

In addition, it was noticed that the LDI protein sequence includes a canonical mode 1 14-3-3 binding site [23]. Since 14-3-3 proteins are known to bind and modulate the activity of many enzymes of carbohydrate metabolism [24], the interactions between LDI and 14-3-3 were also studied.

2. Materials and methods

2.1. Plant materials

Barley plants (*Hordeum vulgare* L.) variety "Golden Promise" were grown in an environmentally controlled growth chamber at 14 °C under a 16 h light/8 h dark cycle at light levels of approximately 450 μE at head height (Philips Son-T Agro 400) and 60–80% relative humidity. Alternatively, "Golden Promise" was grown in the field at Gogar Bank Farm, Edinburgh (in 2005 and 2006). Mature grains were germinated by placing ripe barley grains in a glass Petri dish with a Whatman No. 1 filter paper and 5 ml H_2O followed by incubation at 20 °C in a growth chamber.

2.2. LDI cDNA cloning

Total RNA was extracted from barley grains at 2–4 weeks post anthesis with a LiCl method [25]. Two micrograms of total RNA was used to synthesize 30 μl first strand cDNA using random hexamer primers (Roche) and M-MLV reverse transcriptase (Promega). A 3 μl aliquot of the cDNA product was used in a standard PCR reaction containing 8% (v/v) DMSO and the primers Inhib-5 (5′-ACCAATAAACTAGTATCAACAATGG-CATCCGACCA-3′) and Inhib-6 (5′-CCAACCTTTTTTATT-CATCAATCGGCCACA-3′), using Taq polymerase (Bioline) and 35 cycles of 94 °C for 30 s, 63.5 °C for 30 s and 72 °C for 1 min. The predicted product length was 623 bp. The amplified product was cloned into the pGEM-T Easy vector (Promega) and verified by sequencing of multiple independent clones.

2.3. LDI gene cloning

A sub-genomic *SpeI/XhoI* library was prepared in the pBluescript IIKS vector (Stratagene) from barley DNA isolated from leaves using the Phytopure plant DNA extraction Kit (GE Healthcare). The restriction sites *Spe*I and *Xho*I occur in the 5′- and 3′-untranslated regions respectively of the *LDI* cDNA. After colony blotting the library to nylon membranes, the membranes were hybridized against a PCR-generated digoxigenin-labeled *LDI* probe (10 ng ml^{-1}) in DIG Easy Hyb buffer (Roche). Probe detection was carried out as recommended by Roche for using digoxigenin-labeled PCR-generated probes using CDP-Star as a substrate (Sigma).

2.4. Southern blotting

Genomic DNA (10 μg per sample) was digested with restriction enzymes and separated on a 0.7% TAE agarose gel. DNA was transferred to a nylon membrane using downward capillary blotting [26]. After transfer the DNA gel blot was UV-crosslinked and hybridized against a PCR-generated digoxigenin-labeled *LDI* probe as described above. The blot was washed in 0.2× SSC, 0.1% SDS at 65 °C for 30 min prior to detection of the bound probe.

2.5. Northern blotting

Five micrograms of total RNA per sample was separated on a 1% denaturing agarose gel containing 10 ml 37% formaldehyde per 100 ml gel. After electrophoresis, the gel was blotted onto nylon membranes using a downward capillary blotting procedure [27]. The RNA gel blot was UV-crosslinked and hybridized with a single-stranded digoxigenin-labeled antisense *LDI* RNA probe (100 ng ml^{-1}). Detection steps were carried out as described for DNA gel blots and according to manufacturers' instructions (Roche).

2.6. 3′-RACE

For 3′-RACE, cDNA was synthesised in a reaction containing 2 μg total RNA and oligo d(T) anchor primer (5′-GAC-CACGCGTATCGATGTCGATTTTTTTTTTTTTTTV-3′), 1 μl M-MLV reverse transcriptase (Promega) in a total volume of 20 μl. Two microliters of this cDNA was used in a standard PCR reaction using primers Inhib5 and PCR anchor primer

(5′-GACCACGCGTATCGATGTCGAC-3′). For amplification of tubulin (accession no. X99623), primers Hv-Tub F (5′-TACCACCTCCCTGAGGTTG-3′) and PCR anchor primer were used.

2.7. Genome walking

Genome walking was carried out as described by [28]. About 2.5 μg of genomic barley DNA were digested with either *Sca*I, *Pvu*II, *Eco*RV, *Sma*I or *Dra*I, and ligated to an asymmetric adapter. The adapter libraries were diluted to 200 μl with TE buffer and 1 μl used as a template for a PCR reaction with the adapter primer AP1 (5′-GTAATACGACTCACTATAGGGC-3′) and the *LDI* gene-specific GSP1 (5′-AGACCCGTTTTAT-CACGTAGGTGTGG-3′). One microliter of this PCR reaction was used as a template for the secondary (nested) PCR using primers AP2 (5′-ACTATAGGGCACGCGTGGT-3′) and the *LDI* gene-specific GW1 (5′-AGACGGCGCCGGAGAGGAC-GAAGCGA-3′). The program used for the first PCR comprised 3 min at 94 °C, 7 cycles of 2 s at 94 °C and 3 min at 72 °C, followed by 32 cycles of 2 s at 94 °C and 3 min at 67 °C, finalized by 4 min at 67 °C. The second (nested) PCR program consisted of 3 min at 94 °C, 7 cycles of 2 s at 94 °C and 3 min at 70 °C, followed by 32 cycles of 2 s at 94 °C and 3 min at 65 °C finalized by 4 min at 67 °C.

The *Sma*I and *Pvu*II fragment-based PCR reactions resulted in PCR products of approximately 800 and 600 bp, respectively, which were cloned into the pCR4-Topo vectors (Invitrogen), and one representative clone of each sequenced. This showed both clones to derive from the same 5′-sequence upstream of the *LDI* gene. In order to confirm the sequence of this region, primer LDI-PI (in the promoter sequence, 5′-TTTCACCGACAAAATATTGCT-3′), and primer LDI-PII (overlapping the 5′-untranslated region of the cDNA and the promoter sequence, 5′-TGGTTCAATCTCTTGGCCTGG-3′) were designed and used to amplify the *LDI* upstream sequence from genomic DNA. The sequence of the resulting PCR product was determined.

2.8. Western blotting

Proteins were separated by SDS-PAGE on 12% (w/v) polyacrylamide resolving gels [29]. When duplicate gels were run, one gel was stained by silver-staining or Coomassie blue staining and proteins from the replicate gel were transferred to a nitrocellulose membrane (Amersham-Pharmacia Biotech). Blotted membranes were incubated with polyclonal rabbit antiserum raised against purified LDI from mature barley grains, or polyclonal sheep antiserum raised against spinach 14-3-3. The immunoreactive protein bands were detected by incubation with goat-antirabbit antiserum or donkey–anti-sheep antiserum conjugated to alkaline phosphatase (Sigma) and enzyme activity detected with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). We would like to thank A.E. MacGregor and S.L. Bazin for the LDI antibody, and C. MacKintosh for the 14-3-3 antibody.

2.9. Immunolocalisation

Developing barley grains at 3–4 weeks post anthesis had three to four shallow transverse cuts made across the pericarp before fixation in 3.7% (v/v) formalin:50% (v/v) ethanol:5% (v/v) acetic acid for 48 h. The grains were dehydrated and wax embedded in an automatic processor (Shandon Histocenter) prior to sectioning at 7 μm thickness and mounting on poly L-lysine-coated slides and de-waxing. Slides were then incubated at 65 °C for 1 h in order to reduce endogenous alkaline phosphatase activity. After blocking with 1% (w/v) casein in PBS, slides were incubated for 30 min with anti-LDI antiserum or non-immune serum at 1:500 dilution in blocking buffer, washed three times for 5 min in PBS, 0.1% (v/v) Tween-20, and incubated for 30 min with goat-anti rabbit antiserum conjugated to alkaline phosphatase (Sigma) at 1:50 dilution in blocking buffer. The sections were washed three times as before and enzyme activity detected with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). Photomicrographs were made using an Axiophot microscope (Zeiss).

2.10. Partial purification of limit dextrinase

LD was partially purified from 500 g of unkilned 9-day-old barley malt from variety Alexis with a protocol as described by [30], up to the ion exchange chromatography step, by using a fast protein liquid chromatography system (FPLC, Amersham Pharmacia Biotech). Fully purified LD is unstable, thus partially purified LD was used. The activity of the LD was determined using the Limit-Dextrizyme method (Megazyme) according to manufacturers' recommendations.

2.11. Assay of LDI activity

LDI activity was determined in partially purified extracts as described by [6]. Five developing or germinated grains were extracted with 1 ml of 0.1 M sodium acetate (pH 5.5) containing 10 mM 1,10-phenanthroline as a protease inhibitor [31] per 100 mg fresh weight, at 4 °C for 30 min. The extract was heated to 70 °C for 40 min to precipitate out heat-labile proteins (including endogenous LD), centrifuged at 10,000 × *g* for 5 min and the supernatant retained. The protein content of the LDI extracts was measured using a Bradford assay (BioRad) according to manufacturers instructions. About 10 μg protein of the LDI extract were mixed with 4.3 mU of partially purified LD and the volume made up to 0.5 ml in 0.1 M maleic acid, 0.02% (w/v) Na azide (pH 5.5). The mixture was left to stand for 1 h at room temperature and then assayed with the Limit-Dextrizyme method (Megazyme) according to manufacturers' recommendations. The remaining percentage of LD activity is a function of LDI inhibitory activity. Extracts from germinating grains prepared as described above were also passed through 9 ml Sephadex PD-10 desalting columns (GE Healthcare), the protein containing fractions retained and assayed in parallel with non-fractionated extract. About 10 μg protein aliquots of the LDI extract from mature grains were also incubated for 1 h with 5 μg of purified recombinant barley

14-3-3A, B or C (see following paragraph) or with BSA, prior to assaying LD inhibition as described above.

2.12. 14-3-3 affinity chromatography

Purified recombinant barley 14-3-3A protein (accession no. X62388) and 14-3-3 pull-down affinity chromatography were prepared and carried out as described by [24]. The cDNAs for 14-3-3B (accession no. X93170) and 14-3-3C (accession no. Y14200) were also cloned into pET28B after PCR amplification using primers 14-3-3B pet F (CTGCATATGGCGCAGCC-TGCT), 14-3-3B petR (GTCTCGAGTCCATCTCCAGATTC), 14-3-3C pet F (GTGCATATGTCGGCACCAGG) and 14-3-3C pet R (GCTTGGATCCCCCTCGCTCGA). His-tagged 14-3-3 proteins A, B and C were expressed in *E. coli* strain BL21 DE3 by addition of IPTG to 1 mM, purified over His Gravitrap nickel columns (GE Healthcare) and coupled to CNBr-activated Sepharose (Sigma) at a ratio of 2 mg protein to 1 ml Sepharose slurry. BSA was also coupled to CNBr-activated Sepharose in a similar manner. Extracts were prepared from field-grown developing barley grains (3 weeks post anthesis) by grinding 3 grains/ml extraction buffer (50 mM Tris pH 8, 1 mM PMSF, 1 mM DTT, 1 mM $MgCl_2$), with and without the addition of 1 mM sodium vanadate and 50 mm sodium fluoride. These latter act as protein phosphatase inhibitors, required since 14-3-3 generally binds over a phosphorylated motif in the client protein [23]. The extracts were centrifuged at 10,000 × *g* for 30 min, and 10 U calf intestinal alkaline phosphatase (Fermentas) was added to each milliliter of the extract supernatent without phosphatase inhibitors, and both extracts incubated for 30 min at 37 °C. About 100 μl Sepharose slurry was added to 1 ml of extract, gently rotated at room temperature for 1 h and the Sepharose extensively washed with 50 mM Tris pH 8, 50 mM NaF and 500 mM NaCl. Bound proteins were eluted by addition of 50 μl SDS-PAGE sample buffer to the Sepharose, and heating to 95 °C for 5 min prior to Western blotting using LDI antiserum. We are grateful to P. Schoonheim for sending us cDNA constructs for barley 14-3-3B and 14-3-3C.

3. Results and discussion

3.1. Southern blotting, sequence analysis, and gene expression analysis

Analysis of barley genomic DNA by Southern blotting, using the *LDI* cDNA as a probe (Fig. 1) indicates that *LDI* comprises of a small multigene family. This is in contrast to the situation for LD, which is encoded for by one single gene [13,32]. At least three major hybridising bands are seen for each of the digests, as well as a number of fainter bands. Since the sites for the restriction endonuclease enzymes *Eco*RV and *Bam*HI do not occur within in the *LDI* cDNA or gene, and that of *Xho*I only in the 3′-untranslated region, the additional bands seen here do not arise from one single cleaved *LDI* gene but from several different genes.

The LDI proteins belong to a family of small inhibitor proteins, called the cereal trypsin/α-amylase inhibitor family

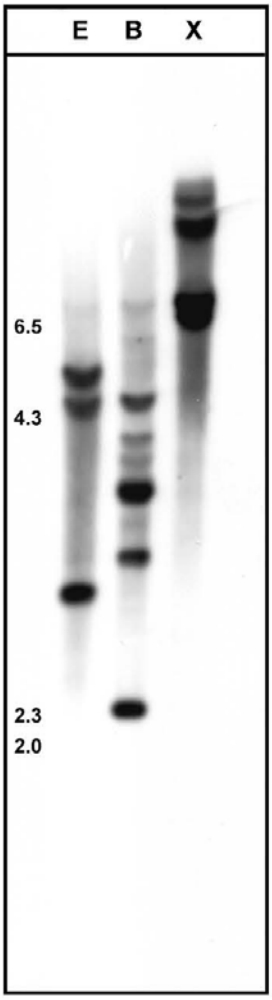

Fig. 1. Southern analysis of the *LDI* gene in wild-type genomic barley DNA. DNA was digested with E: *Eco*RV, B: *Bam*HI and X: *Xho*I, fractionated, blotted and hybridised with a digoxigenin-labelled *LDI* cDNA probe. Molecular weight marker sizes in kilobasepairs.

[33] but the other members of this family differ significantly from LDI in sequence at the amino acid level (for example only 48% identity over 105 amino acids with the barley trypsin inhibitor, accession no. X 17302) and there is essentially no homology at the nucleotide level, thus it is improbable that any of the major bands seen by Southern blotting correspond to these trypsin/α-amylase inhibitor genes. Whether all of the *LDI* genes are expressed is not yet known, however interrogation of the Institute for Genomic Research (http://www.tigr.org) barley EST database did not reveal any expressed sequence tags other than essentially identical matches to the known *LDI* cDNA sequence, which means that these additional *LDI* genes are

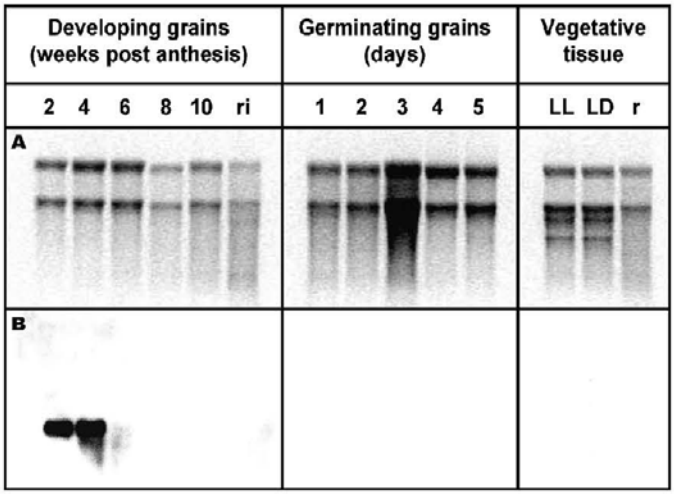

Fig. 2. Northern analysis of *LDI* gene expression. (A) RNA gel; (B) exposed X-ray film; ri: ripe grains; LL: leaves light; LD: leaves dark; r: roots. Fractionated, blotted total RNA (5 μg) was hybridised with a digoxigenin-labelled *LDI* cDNA riboprobe.

either very similar to each other in sequence, or not all expressed.

Sequence analysis of the barley *LDI* cDNA from cv Golden Promise isolated here (accession no. DQ285565) shows an almost identical sequence to that of cv Bomi (accession no. XI3443) [8] with five nucleotide exchanges, three of which are in the coding region. This results in two amino acid differences between the derived protein sequences of cv Bomi and cv Golden Promise (P for L at position 35, and E for A at position 136), and no differences between Golden Promise and the sequenced protein of cv Harrington as reported by MacGregor (not including the sequences missing from cv Harrington at the N and C termini) [6]. Sequence analysis of the *LDI* genomic DNA from Golden Promise (accession no. DQ285564) showed this to be the source of the cDNA reported here, since all of the coding region and both the 3′- and 5′-untranslated regions were identical to that of the cDNA. The *LDI* genomic sequence contains no introns.

When MacGregor et al. first characterised the LDI protein, they purified two major proteins of identical amino acid sequence [6]. The difference in p*I* of the two forms of inhibitor was attributed to differential post-translational modification; the high p*I* (7.2) form may have a covalently bound cysteine residue whereas the low p*I* (6.7) form has a glutathione residue attached and it was suggested that both these proteins are encoded for by a single gene [9]. However, given the existence of an *LDI* multigene family, the possibility still remains open that these proteins are encoded for by different genes, especially since 24 amino acids comprising the N-terminal signal sequence and 9 C-terminal amino acids were not determined, and thus could differ between the high and low p*I* forms.

In order to investigate the time and place of *LDI* gene expression, total RNA was extracted from a developmental series of grains, from germinating grains and from seedling, leaves and roots, and was hybridised with an *LDI* cDNA-derived riboprobe (Fig. 2). The probe hybridised with RNA extracted from grains at 2 and 4 weeks post anthesis in developing grains but not after this time, nor with RNA from germinating grains or other tissues, indicating that *LDI* is expressed exclusively in developing grains, and is not associated with chloroplast starch metabolism in leaf material. *LDI* gene expression in different tissues was also analysed using RT-PCR. The 3′-RACE technique, using an anchored oligo dT primer was used in order to avoid amplification of contaminating genomic DNA, since the genomic sequence contains no introns and is thus indistinguishable from the cDNA. The results of 3′-RACE on developing grains, endosperm, aleurone and leaves are shown in Fig. 3. No *LDI* gene expression was detected, as expected, in the negative control and genomic DNA control. *LDI* gene expression was found in whole developing grains at 2 and 4 weeks post anthesis and in the endosperm. No *LDI* expression was found in the pericarp (which includes the aleurone layer) at 2 days after germination, nor in leaves. This data confirms that obtained from Northern blotting, and shows that *LDI* gene expression is confined to the endosperm. The timing of *LDI* gene expression is consistent with its location; the endosperm is physiologically dead at maturity thus *LDI* must be expressed during early development of the grain.

3.2. Genome walking

A 797 bp sequence of the upstream region from the *LDI* gene was isolated by genome walking. Bioinformatic analysis of this promoter sequence (accession no. DQ285564) using the Web Signal Scan program [34] revealed several potential plant DNA regulatory elements. As well as elements common to most plant genes such as the CAAT and TATA boxes, other elements were

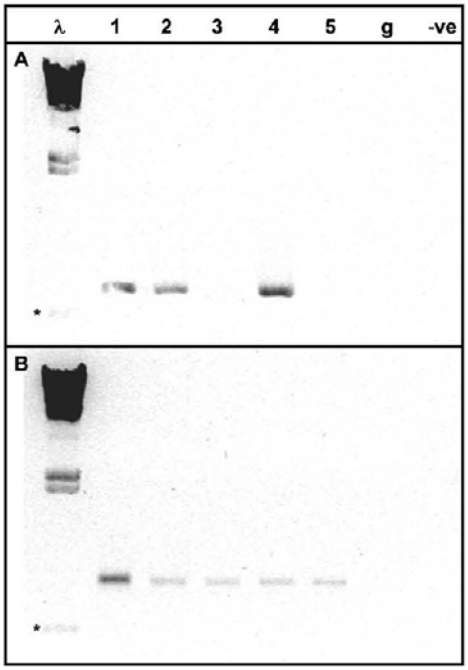

Fig. 3. 3′-RACE analysis of *LDI* gene expression. 3′-RACE PCR with *LDI*-specific primer Inhib5 and PCR anchor primer (A) and primers Hv-Tub F + PCR anchor primer specific for the tubulin gene (B) on cDNA derived from different tissues. λ: λ/*Hin*dIII molecular weight marker, the asterisk shows the position of the 564 bp band; 1: grains 2 wpa; 2: grains 4 wpa; 3: aleurone after 2 days of germination; 4: endosperm 2 wpa; 5: leaves after 8 h light; g: genomic DNA control; −ve: negative control PCR; wpa: weeks post anthesis.

found that are also present in the promoters of genes expressed in cereal grains. These elements include 10 copies of AAAG, a core site required for binding of endosperm-specific Dof proteins in maize [35] and TGHAAARK, an element present in the promoter of the B-hordein gene of barley and the α-gliadin, γ-gliadin, and low molecular weight glutenin genes of wheat [36]. The presence of such elements is in accordance with our data showing *LDI* gene expression in the endosperm.

3.3. Immunolocalisation and LDI activity

Immunolocalisation studies on 3–4-week-old developing grains showed a signal for LDI predominantly in the outer section of the endosperm (Fig. 4). The location of immunologically reactive LDI protein is consistent with the expression pattern of the *LDI* gene. Partially purified LDI extracts were prepared from developing and germinating growth room-grown grains of Golden Promise, and used for both Western blotting and activity assays of the same extracts (Fig. 5). An immunologically reactive signal corresponding to the molecular weight of LDI was seen in developing grains at 4 weeks post anthesis onward (Fig. 5B), which agrees with the timing of *LDI* gene expression. LDI protein levels remained constant throughout grain development and started to decrease in germinating grain after 2 days (Fig. 5D). The inhibitory activity of extracts toward LD reached a maximum during early grain development, and decreased rapidly during germination, mirroring the decrease in LDI protein. However, by day 5, extracts showed higher levels of LD activity than that added to the controls (Fig. 5E). This is not due to the LD that is produced during germination, since the extracts were heated during preparation in order to denature any LD (which also results in relatively poor resolution of these gels). Small maltodextrins are known to strongly promote a transglycosylation activity of LD, which is also detected by the Limit-Dextrizyme method used to assay LD debranching activity [37,38]. Such dextrins would be present in the germination extracts, since they are released by starch degrading enzymes during germination and this would account for the apparent increase in LD activity during germination. In order to test this hypothesis, extracts from germinating grains (2005 field-grown harvest) were passed through a de-salting column in order to remove low molecular weight compounds prior to assay. As can be seen in Fig. 6, the enhancement of LD activity by these extracts is substantially reduced by this treatment, in agreement with a role for dextrins in this process.

3.4. A role for LDI in starch grain development?

As outlined in Section 1, a considerable body of evidence implicates debranching enzyme activity in the biosynthesis of

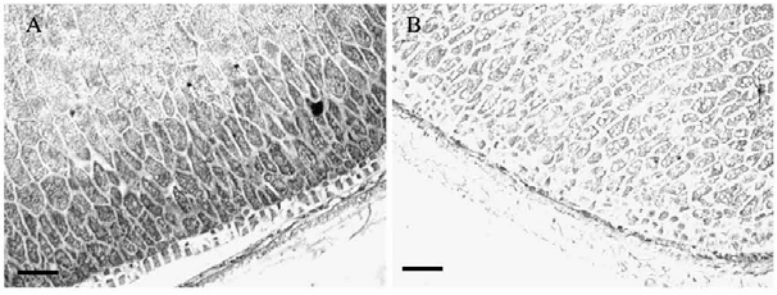

Fig. 4. Immunohistochemical localisation of LDI. Developing barley grains 3–4 weeks post anthesis were fixed, sectioned and incubated with antisera. (A) Immunostaining with LDI antiserum and (B) non-immune antiserum. The bar is 100 μM long.

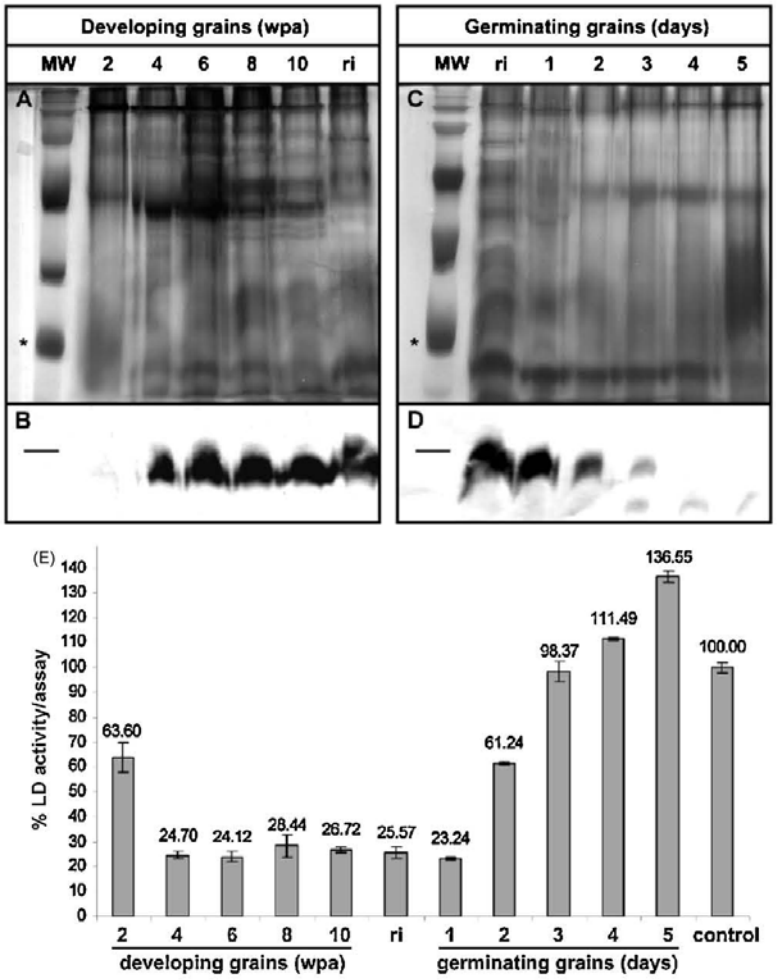

Fig. 5. LDI presence and activity in developing and germinating grains. (A + C) silver-stained SDS-PAGE of partially purified LDI extracts from developing (A) and germinating (C) grains corresponding to 5 μg total protein; asterisks indicate the 21 kDa marker bands; (B + D) immunoblot developed with LDI antiserum, bars indicate the 21 kDa marker bands; (E) LDI activity assay. The control (100%) represents the amount of LD used for each assay. LDI extracts corresponding to 10 μg total protein were mixed with LD and assayed for the remaining free LD activity. Each value represents the mean ± standard error of three replicate experiments. MW: molecular weight marker sizes in kDa are 97, 66, 45, 31, 21 and 14; ri: ripe grain; wpa: weeks post anthesis.

amylopectin and in the formation of starch granules (reviewed [14]). Whilst the genetic lesions resulting in reduced branching enzyme activity in higher plants have all been shown to be in the gene encoding isoamylase, the maize and rice isoamylase mutants are pleiotropic, and have reduced LD activity, and combined isoamylase and LD mutations in maize have additive effects, making an unambiguous assignation of function difficult. The barley starchy endosperm differs in its properties across the grain; starch grains of the endosperm are of two types, large (A) and small (B), and the outer layers of the endosperm contain many more B-type starch grains than in the inner regions. The larger A-type granules are nucleated at around 5 days post anthesis, whereas the B granules arise later at around 14–21 days post anthesis from protrusions emanating from the A granule amyloplasts [39,40], at about the same time and place in the endosperm as LDI is expressed. The spatial and temporal correlation of LDI gene and protein expression with the nucleation of the B-type starch grains within the endosperm, taken together with the finding that antisense inhibition of *LDI* expression results in barley grains with much reduced numbers of B-type grains [22], suggests that LDI may play a role in the synthesis of the B-type grains. Since LD, the target for inhibition by LDI, is also known to be expressed and active in the developing grain between 6 and 19 days post anthesis [32], a possible function of LDI may be to prevent LD from degrading potential dextrin primers for starch synthesis, or granule

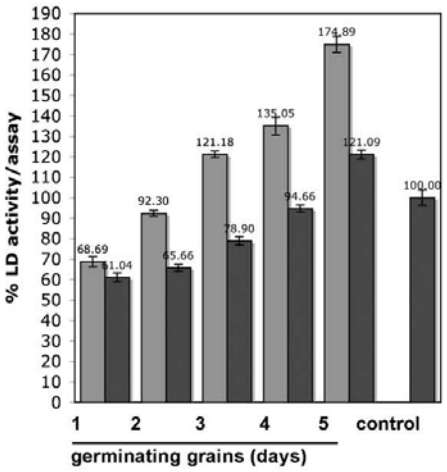

Fig. 6. Reduction in the stimulation of LD activity by gel filtration of extracts. LDI activity assays of germinating grain extracts before (grey bars) and after gel filtration through a de-salting column (black bars). The control (100%) represents the amount of LD used for each assay. LDI extracts corresponding to 10 μg total protein were mixed with LD and assayed for the remaining free LD activity. Each value represents the mean ± standard error of three replicate experiments.

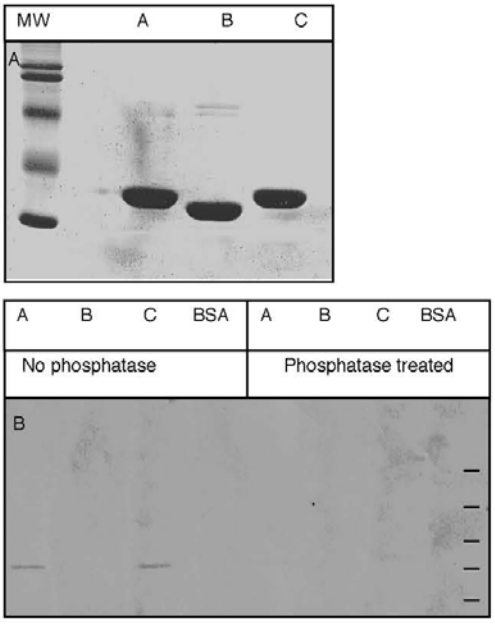

Fig. 7. Interaction between 14-3-3 and LDI. (A) SDS-PAGE analysis of 5 μg recombinant barley 14-3-3A, B and C proteins used in pull-down assays. MW, molecular weight markers at 116, 97, 66, 45 and 29 kDa. (B) 14-3-3 affinity pull-downs using extracts from developing barley grain at 3 weeks post anthesis (plus and minus phosphatase treatment) were immunoblotted using LDI antiserum. A, 14-3-3A, B, 14-3-3B, C, 14-3-3C, BSA, immobilised BSA. Bars indicate molecular weight markers at 45, 29, 20, 14 and 6 kDa.

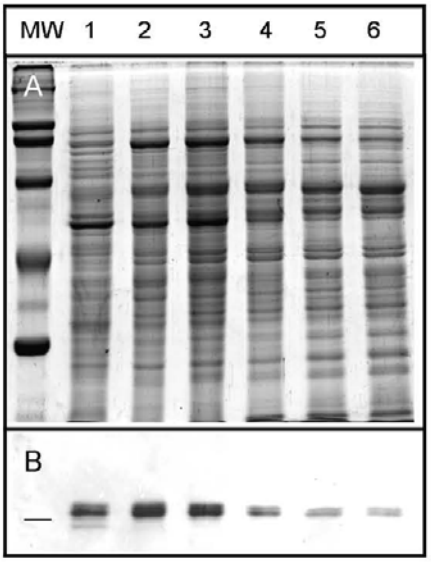

Fig. 8. Western analysis of 14-3-3 protein in endosperm tissue during development. (A) SDS-PAGE fractionated endosperm protein from developing barley grain at different weeks post anthesis (1–6). MW, molecular weight markers at 116, 97, 66, 45 and 29 kDa. (B) Immunoblot developed with 14-3-3 antiserum, the bar indicates the 29 kDa marker band.

crystallisation. However until further evidence is available (for example, analysis of a barley LD mutant), such a mechanism must remain speculative.

3.5. 14-3-3 affinity pull-down

During our analysis of LDI, it was noted that LDI contained a mode-1 14-3-3 binding site ((R/KXXSXP) [23] at amino acids 58–63 (RGPSRP). 14-3-3 proteins are ubiquitous 30 kDa eukaryotic proteins that bind to specific phosphorylated domains of target proteins that include signalling proteins and metabolic enzymes [41]. The capacity of 14-3-3 to bind to LDI was investigated; three well-characterised barley 14-3-3 isoforms [42], the 14-3-3 A, B and C proteins were over-expressed in *E. coli* (Fig. 7A), immobilised to activated Sepharose and used in affinity pull-down assays with extracts from 3 to 4 week post anthesis field-grown barley grains, followed by SDS-PAGE and Western blotting (Fig. 7B). An immunologically reactive signal for LDI was seen in proteins bound to immobilised 14-3-3A and 14-3-3C, but not to 14-3-3B, nor to the control BSA-Sepharose. Treatment of extracts with alkaline phosphatase abolished LDI binding to 14-3-3. Thus, LDI binding is 14-3-3 sub-type-specific, and dependent on phosphorylation. A Western blot analysis of proteins extracted from endosperm during a developmental series of field-grown Golden Promise barley showed 14-3-3 proteins to be present in the endosperm throughout grain development (Fig. 8), thus LDI and 14-3-3 also have the potential to interact *in vivo*.

14-3-3 proteins are known to bind and regulate the activity of a number of enzymes involved in starch metabolism [24], and down-regulation by antisense of an *Arabidopsis thaliana* 14-3-3 gene results in enhanced starch accumulation [43]. The regulation of enzymes of starch metabolism in wheat amyloplasts by phosphorylation and protein–protein interaction has also been reported by Tetlow et al. [44]; it is possible that the inhibition of LD activity by LDI is also modified by 14-3-3 binding. A three-dimensional model of the LDI protein, based on the structure of the ragi α-amylase-trypsin inhibitor [9], shows that the probable 14-3-3 binding site in LDI is readily accessible, being located at the surface of the protein on a loop between α-helixes 1 and 2. This site is not conserved in similar proteins such as the maize and ragi α-amylase-trypsin inhibitors, and coincides with the position of residues involved with trypsin inhibition in these proteins, a function not found in LDI [7].

The ability of recombinant 14-3-3A, B or C to modify LD and LDI activity was tested by pre-incubating barley grain extracts or partially purified LD with 14-3-3 prior to assay for LD activity or inhibition by LDI. However, the addition of 14-3-3 to these assays had no significant influence on either LD or LDI activity (data not shown). It is known that the C-terminal of certain 14-3-3 isoforms is autoinhibitory to function [45], and that barley 14-3-3A is cleaved *in vivo* to remove this inhibitory domain [46]; further studies on C-terminal truncated barley 14-3-3 proteins may show modification of activity of LDI or LD. Alternatively, LDI might have other functions altogether that are regulated by 14-3-3.

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